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13. ABSTRACT (Maximum 200 Words) The Bionetics Corporation staffed and maintained laboratories to support red blood cell preservation research for the Blood Research Detachment, Walter Reed Army Institute of Research, initially at 1413 Research Blvd., Rockville, MD 20850 and, after relocation, at Building 504, Walter Reed Forest Glen Annex, Silver Spring, MD 20910. Contract staff completed three <i>in vitro</i> trials. The first trial evaluated an investigational system for processing frozen red cells which would extend the post-thaw shelf life from 1 day to 15 days; the second compared red cells stored in a polyvinyl chloride (PVC) versus non-PVC bags and the third tested a blood sterilization process. A clinical trial, which measured the 24 hour post-transfusion survival of autologous red cells prepared in the investigational system for processing frozen red cells, was nearing completion. Preliminary results indicate fully acceptable survival after 15 days post-thaw storage. One manuscript describing research completed during the period of the previous report was published and two additional manuscripts are in press. The Bionetics Corporation advanced the Blood Research Detachment's mission.				
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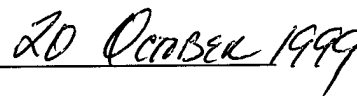
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Table of Contents

Front Cover	1
Report Documentation Page (SF 298)	2
Foreword	3
Table of Contents	4
Introduction	7
A. "Phlebotomy Procedures for Use on Human Subjects" WRAIR #514, HURRAD Log #A-6664.	
Background	10
Methods	10
Results	11
Discussion / Conclusion	12
B. "Evaluation of the <i>in vitro</i> and <i>in vivo</i> Viability of Red Blood Cells Stored in Blood Storage Solutions CPDA-1 for 35 Days and in AS-5 for 42 Days at 1-6 C and Exposed for Short Periods to Higher Temperature" WRAIR #633, HURRAD Log #A-7818.	
Background	13
Methods	13
Results	13
Discussion / Conclusion	13
C. "The <i>In Vitro</i> Storage Characteristics of Red Blood Cells Stored in Increasing Volumes of AS-1"	
Background	14
Methods	14
Results	14
Discussion / Conclusion	14
D. "The Storage Characteristics of Red Blood Cells Stored in Experiment Additive Solutions EAS-61 and EAS-64"	
Background	14
Methods	14
Results	14
Discussion / Conclusion	14

E. "Evaluation of the *in vivo* Viability of Red Blood Cells Stored for Prolonged Period in the Leukotrap® RC AS-24 System" WRAIR #572, HURRAD Log #A-6986.

Background	16
Methods	16
Results	16
Discussion / Conclusion	16

F. "Evaluation of the *In Vivo* Viability of Red Blood Cells Stored for Eight Weeks in the Leukotrap® Whole Blood AS-3 System" WRAIR #591, HURRAD Log #A-7089.

Background	16
Methods	16
Results	16
Discussion / Conclusion	16

G. "Evaluation of *in vitro* Storage of Human Red Blood Cells after Sterile Frozen Storage, Processing on Haemonetics 215 and 2 Weeks of Liquid Storage in AS-3" WRAIR #714.

Background	17
Methods	17
Results	18
Discussion/Conclusion	18

H. "Evaluation of *in vivo* Viability of Human Red Blood Cells after Sterile Frozen Storage and Processing on the Haemonetics 215 and 2-Week liquid Storage in AS-3" WRAIR #715, Log #A-8654.

Background	17
Methods	17
Results	18
Discussion/Conclusion	18

I. "*In Vitro* Comparison of Red Blood Cells Stored in EAS-61 in Polyolefin Versus Polyvinyl Chloride Storage Bags.

Background	19
Methods	19
Results	20
Discussion/Conclusion	21

J. "Photochemical Treatment of Platelet Concentrates Infected with Ehrlichia Chaffeensis"
WRAIR #731.

Background	21
Methods	22
Results	23
Discussion/Conclusion	24

K. "Evaluation of the *In Vivo* Viability of Red Blood Cells after 8- and 9-Week Storage in Terumo's EAS-61 Additive Solution" WRAIR #713, Log #A-8655.

Status	24
General and Administrative	24
Conclusion	25
References	26

Appendices

1. List of Personnel	29
2. Manuscript: "The Viability of Autologous Human Red Cells Stored in Additive Solution 5 and Exposed to 25°C for 24 Hours"	30
3. Manuscript: "The Effects of Phosphate, pH, and Additive Solution Volume on RBCs Stored in Saline-Adenine-Glucose-Mannitol Additive Solutions"	37

4. Manuscript: "A Hypotonic Storage Solution Did Not Prolong the Viability of Red Blood Cells"	59
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ANNUAL REPORT: DAMD94-C-4154

21 September 1998 - 20 September 1999

INTRODUCTION

Nature of the Problem:

Because combat is synonymous with bloodshed and blood replacement saves lives, the US Army Medical Research and Materiel Command maintains facilities and programs to develop improved blood products.

The Background of the Previous Work:

The US Army has, for decades, conducted research in red blood cell preservation and the production of acellular hemoglobin solutions for use in combat casualty care. From 1974 through 1992, that research took place at the Letterman Army Institute of Research (LAIR) located at the Presidio. The LAIR facility was closed as the result of Base Realignment and Closure actions and the Blood Research Detachment was relocated to leased laboratory space at 1413 Research Blvd., Rockville, Maryland. On 19 September 1994 The Bionetics Corporation (TBC) was awarded a contract to operate and maintain equipment and provide technical support to the Blood Research Detachment (BRD). For the first two years of the contract, the contractors supported the Hemoglobin Production Facility (HPF) and integral analytical chemistry laboratory which provided quality control and characterization testing. The HPF produced several hundred liters of a high-purity hemoglobin-based blood substitute material for research. The acellular hemoglobin solutions manufactured by the HPF were based on biochemical modification of stroma-free hemoglobin as described in the literature.^{1,2,3,4} A detailed description of the hemoglobin solution production process and process improvements is contained in a manuscript, published in October 1997 by the journal "Biologics", the journal of the International Association of Biological Standardization. A reprint copy of the complete journal article, Highsmith FA, Driscoll CM, Chung BC, Chavez MD, Macdonald VM, Manning JM, Lippert LE, Berger RL, and Hess JR. "An Improved Process for the Production of Sterile Modified Haemoglobin Solutions"

Biologics, 1997;25:257-268, was included in the previous annual report. During the last several months of HPF operation, the contract staff also supported the US Navy liposome encapsulated hemoglobin research.^{5,6} HPF related activities were terminated on 20 September 1996 and the facility closed. Since then, the production facility equipment was either turned-in for disposal or transferred to other Institute activities for their use.

Contract staff has continuously operated and maintained the blood cell preservation research of the Blood Storage Laboratory (BSL), a fully equipped red cell research laboratory currently staffed by 3 full-time and 2 part-time employees. One of the Blood Banking Specialist members of this team is also the Project Manager of the contract and the contract designated Principle Investigator holds the specialty certification in Blood Banking. Since the last report a technologist has joined the contract team who also holds the same certification. A list of contract staff as of the close of the FY is included at Appendix 1.

The current Food and Drug Administration (FDA) licensed anticoagulant, preservative solutions allow storage of red blood cells at 4° C for 42 days after collection. Work by Meryman *et. al.*^{7,8} and Greenwalt, Dumaswala and colleagues^{9,10} indicates potential for extended storage. Greenwalt and colleagues have recently developed an experimental additive solution which preserves red cells for 56 days with average red cell survival of at least 75%.¹¹ Subsequent modifications have yielded greater than 75% 24-hour post transfusion survival after 10 week of storage.¹²

It is estimated that one half million units of blood expire per year in the United States. It is further estimated that if expiration were extended to 8 weeks, one-third of the expired units now discarded would be transfused.¹³ Extended shelf-life of liquid stored blood would have significant utility to the Armed Services Blood Program as it supports the Department of Defense blood transfusion requirements world-wide. The major advantages of an eight-week extended shelf life red cell product include, reducing resupply requirements by up to 30%, reducing the blood collection requirements to replace outdated products, and increasing the practicality of rotating aged but unexpired red cell products from the theater of operations to continental U.S. (CONUS) treatment facilities where they are more likely to be used. Extending the storage time to 12 weeks would halve the resource requirements. The net result is reduced costs through enhanced utilization of a scarce, perishable resource.

The Purpose of the Present Work:

The BSL evaluates the effectiveness of candidate red blood cell anticoagulant preservative systems and storage strategies along with their potential for further development. Much of the work is done in conjunction with other principal investigators in the BRD. The BRD supports the Military Blood Program by providing data which will evaluate the safety, effectiveness, and practicality of new products or procedures related to the collection, processing and distribution of red blood cells.

During this reporting period, the BSL has performed research under two clinical trial protocols, a phlebotomy type protocol which permits collection of blood from volunteers for *in vitro* research and three *in vitro* research projects. A publication, describing research on two clinical trial protocols completed in a previous FY was accepted for publication and initiation of another clinical trial awaits the availability of the commercially manufactured investigational blood storage system. The BACKGROUND, METHODS, RESULTS, and DISCUSSION / CONCLUSION from each research effort will be described separately in order to maximize continuity. A short description of the status of each project begins the description of each protocol. If a manuscript has either been submitted for publication or published, the abstract from the manuscript is reproduced in the body of the report and the entire manuscript or copy of the published journal article is added to the report as an appendix.

The phlebotomy type protocol activities will be described first, followed by descriptions of the clinical trials, and the *in vitro* projects (in the order each protocol was initiated). The ultimate goal of each clinical trial is to determine if the mean 24 hour post-transfusion red cell survival at the end of the storage period exceeds 75% in a minimum of ten volunteers. Furthermore, at least 99% of the cells collected must also remain intact on the final day of storage. In nearly all instances, a preliminary *in vitro* experiment or set of experiments is conducted to indirectly evaluate the quality of blood stored before the clinical trial and human red cell survival studies are initiated.

Description of research conducted:

A. "Phlebotomy Procedures for Use on Human Subjects" WRAIR #514, HURRAD Log #A-6664.

BACKGROUND

Aspects of red blood cell physiology critical to blood storage are species specific; therefore valid *in vitro* studies of the red blood cell storage lesion require freshly collected human blood. The quantities required range from as little as 3.0 mL to as much as a full unit, 450 mL.

METHODS

Volunteers are recruited from within the Detachment, other tenants of the Gillette Building, Building 503, Forest Glen and the immediately surrounding community and informed fully as to the risks of donation. Potential volunteers were screened for anemia, transfusion transmitted diseases (TTD) and medical conditions which would make blood donation unsafe using the criteria of the American Association of Blood Banks¹⁴ and the Food and Drug Administration 21CFR640.¹⁵ The total amount of blood collected in an 8 week period is limited to 525 mL. A physician certified in Advance Cardiac Life Support was present at all full unit phlebotomies. Volunteers are compensated for their blood donations IAW 24 USC 30 and AR 40-2.

A personal computer data base is used to maintain documentation of all volunteer related transactions and assure compliance with donation volume and interval limitations. Phlebotomies were performed by trained contract staff and selected, trained active duty personnel.

RESULTS

During fiscal year 1999, the following blood collections were made.

Volume	WRAIR	BRD / Clinical Trial	Anthrax Immune / Pittman	Transfusion Med Rsch	AFIP / AFDIL	Total
1 - 50 mL	102	32	N/A	4	2	140
51 - 100 mL	22	N/A	N/A	3	0	22
101 - 200 mL	6	N/A	N/A	0	1	7
Units (>200mL)	30	16	95	7	4	152
Total Number of Phlebotomies Performed						321

The set of collections labeled "WRAIR" were performed for various investigators within the Blood Research Detachment and the "BRD/Clinical Trial" collections were made specifically in support of clinical trial protocols. The collections identified as "Anthrax Immune / Pittman" were collected in collaboration with LTC Phillip Pittman, MC, USAMRIID. The plasma from these anthrax immunized volunteers was used in the development of an anthrax immune globulin; the red cells for these collections were used in other BRD research. The collections labeled "Transfusion Med Rsch" were performed to support research in the Transfusion Medicine Research Laboratory of the Naval Medical Research Institute which had been co-located with the BRD. The remaining seven collections were in support of the Armed Forces DNA Identification Laboratory of the Armed Forces Institute of Pathology which also had been co-located with the BRD until our relocation in March 1999.

Eighty-seven new volunteers were enrolled during the fiscal year. The majority of the newly enrolled volunteers had blood collected only for the harvest of their anthrax-immune plasma. Twenty-four of the enrolled volunteers were converted to inactive status because they had left the area and were otherwise unavailable or withdrew for unstated personal reasons; five additional volunteers were permanently deferred because of a disqualifying condition. One hundred eighty-three sets of transfusion transmitted disease tests were performed during the report period. Two separate reactive results for anti-hepatitis B core antibody, and a single reactive result for the

antibody to human immunodeficiency virus (HIV) I/II were detected. The affected volunteers were informed of the test results and counseled by a physician. The volunteer whose blood tested reactive for anti-HIV I/II had received an experimental HIV vaccine; therefore the positive test result was expected.

A single episode of donor syncope occurred during the 321 phlebotomies. Such an event occurs occasionally during blood donation and is not unusual. The staff employed the procedures specified in the protocol to deal with donor reactions; there were no sequelae.

DISCUSSION / CONCLUSION

Ninety-three percent of the collections supported WRAIR and USAMRMC sponsored research. The remaining seven percent of collections were performed for the Transfusion Medicine Research Laboratory of the Naval Medical Research Institute and the Armed Forces Institute of Pathology co-located with the BRD. The blood collection requirements of the BRD were safely provided from a pool of healthy, screened volunteers.

B. "Evaluation of the *in vitro* and *in vivo* Viability of Red Blood Cells Stored in Blood Storage Solutions CPDA-1 for 35 Days and in AS-5 for 42 Days at 1-6 C and Exposed for Short Periods to Higher Temperature" WRAIR #633, HURRAD Log #A-7818.

STATUS

This clinical trial was completed during the previous FY. A manuscript was submitted for publication in TRANSFUSION, the journal of the American Association of Blood Banks, and subsequently published in the September 1999 issue.

ABSTRACT

No data exist on the viability of red cells (RBCs) stored in modern additive solution systems allowed to warm above 10°C. In a randomized crossover study, 3 units of blood were collected at least 8 weeks apart from 11 volunteer donors and stored in additive solution 5 (AS-5). Of 3 units from each volunteer, 1 was stored for 6 weeks at 4°C, 1 for 5 weeks at 4°C except for 24 hours at 25°C on Day 14, and 1 for 5 weeks at 4°C except for 24 hours at 25°C on Day 28.

Units were sampled periodically during storage; at the end of storage, viability was measured by the $^{99m}\text{Tc}/^{51}\text{Cr}$ double-label method. RBC viability was not significantly different in the storage protocols. Less than 1 percent of stored cells hemolyzed. RBC ATP concentrations at the end of storage correlated with viability and were approximately equal in the warmed units after 30 days' storage and the conventionally stored units after 42 days. The data suggest that RBCs stored in AS-5 and allowed to warm to 25°C for 24 hours lose about 12 days of their shelf life.

BACKGROUND / METHODS / RESULTS / DISCUSSION / CONCLUSION

A copy of the published manuscript describing the background, methods, results, discussion and conclusion from this clinical trial is attached to this report as Appendix 2.

C. "The *in vitro* storage characteristics of red blood cells stored in increasing volumes of AS-1"

and

D: "The storage characteristics of red blood cells stored in Experiment Additive Solutions EAS-61 and EAS-64"

STATUS

The *in vitro* study of the additive solution AS-1 was completed during the previous FY; the study of the experimental additive solution EAS-61 was completed during the current FY. The description and results of these two studies and a third study involving both EAS-61 and EAS-64, conducted by a collaborator, Dr. Tibor Greenwalt from the University of Cincinnati and Hoxworth Blood Center, were combined in a single manuscript. The manuscript has been accepted for publication in the journal TRANSFUSION; publication date is pending. All three protocols employed the same experimental design in which either three or four individual blood unit blood donations were pooled and mixed. The pooled, mixed blood was divided into either three or four aliquots the number determined by the number of units pooled, each aliquot receiving a different treatment. The treatments differed in either the composition or amount of the additive solution. Because pooling moderates or averages the sometimes significant observed differences in storage characteristics of blood from different volunteers, far more powerful data

can be obtained than when each individual's blood unit is analyzed separately. The data from these experiments, when analyzed together, provided insight into the relative contributions of additive solution volume and additive the supplements mannitol and phosphate.

ABSTRACT

RBC ATP concentrations are the most important correlate of RBC viability. These studies tested whether increased additive solution volume, pH, and phosphate content increased stored RBC ATP concentrations. In three studies, units of blood were pooled in groups of 3 or 4 units and realiquoted as combined units to reduce intra-donor differences. Thirty units were stored in AS-1 at RBC concentrations equivalent to 100, 200, or 300 mL of additive solution. RBCs from 24 units were stored in 100, 200, 300, or 400 mL volumes of an experimental additive solution, EAS-61. Thirty units were stored in 100 mL of AS-5, 200 mL of EAS-61, or 300 mL of EAS-64. RBC ATP concentration and other measures of RBC metabolism and function were measured weekly. RBC ATP concentrations decreased sooner with storage in increasing volumes of AS-1. In EAS-61 and EAS-64, RBC ATP concentrations initially increased and stayed elevated longer with increasing additive solution volume. Addition of disodium phosphate to saline-adenine-glucose-mannitol additive solutions increases the RBC ATP concentrations. Reducing storage hematocrit appears to have an separate beneficial effect in reducing hemolysis.

BACKGROUND / METHODS / RESULTS / DISCUSSION / CONCLUSION

A copy of the accepted manuscript describing the background, methods, results, discussion and conclusion from these two clinical trials is attached to this report as Appendix 3.

E. "Evaluation of the *in vivo* Viability of Red Blood Cells Stored for Prolonged Period in the Leukotrap® RC AS-24 System" WRAIR #572, HURRAD Log #A-6986.

and

F. "Evaluation of the *in vivo* Viability of Red Blood Cells Stored for Eight Weeks in the Leukotrap® Whole Blood AS-3 System" WRAIR #591, HURRAD Log #A-7089.

STATUS:

WRAIR protocols # 572 and # 591 were performed under terms of a cooperative research and development agreement (CRDA) with the MEDSEP Division of the PALL Filter Corporation. Both protocols were completed in a previous fiscal year and individually described in a previous annual report. WRAIR protocol #572 evaluated an experimental system incorporating a new formulation of chemicals already used in FDA approved solutions for blood storage, and an integral white blood cell removal filter; the system is designated AS-24. The objective of the first protocol was to determine if this unique preservative formulation coupled with pre-storage white cell removal would permit storage for eight weeks. WRAIR protocol #591 was designed to evaluate a second experimental system for potential eight week red cell storage. A secondary purpose was to evaluate effects of periodic mixing combined with either horizontal or vertical storage. The blood collection system utilized in protocol # 591 differed from the system employed in protocol # 572 in two aspects. First, the FDA licensed AS-3 replaced the experimental hypotonic AS-24 preservative solution in the test units. Second, the system was reconfigured to accomplish white cell removal during collection of the whole blood rather than after the preparation of the packed cells as was the case in protocol #572. The results from both protocols were combined into a single manuscript which was accepted for publication in the journal TRANSFUSION. The publication date is pending.

ABSTRACT

Hypotonic storage solutions and leukofiltration are both reported to improve RBC viability. This study tested the ability of an investigational hypotonic storage solution, AS-24 (Medsep Corp), to extend the viability of liquid-stored RBC to 8 weeks. In a pair of cross-over trials, 1)

eleven RBC units, leukoreduced by filtration and stored in AS-24 for eight weeks were compared with units from the same donors stored for six weeks in AS-3, and 2) thirteen RBC units, leukoreduced by filtration and stored in AS-3 for eight weeks, were compared with units from the same donors stored for six weeks in AS-3. Viability was measured by the $^{51}\text{Cr}/^{99\text{m}}\text{Tc}$ double isotope method. RBC viability at 8 weeks averaged $64\pm 3\%$ in the AS-24 units and $67\pm 2\%$ in the AS-3 units, but was equal at $77\pm 3\%$ and $77\pm 2\%$ after 6 weeks storage in AS-3 in both trials. Pre-storage leukoreduction and storage in AS-24 did not extend RBC viability to 8 weeks. The improved viability previously demonstrated with storage of dilute suspensions of RBC in hypotonic solutions is probably caused by factors other than the hypotonicity.

BACKGROUND / METHODS / RESULTS / DISCUSSION / CONCLUSION

A copy of the accepted manuscript describing the background, methods, results, discussion and conclusion from these two clinical trials is attached to this report as Appendix 4.

G. "Evaluation of *in vitro* Storage of Human Red Blood Cells after Sterile Frozen Storage, Processing on the Haemonetics 215 and Two Weeks of Liquid Storage in AS-3" WRAIR # 714.

and

H. "Evaluation of *in vivo* viability of human red blood cells after sterile frozen storage and processing on the Haemonetics 215 and 2-week liquid storage in AS-3". WRAIR #715, Log # A-8654.

STATUS:

These two protocols were initiated in order to fulfill our obligations which are part of a Memorandum of Understanding between the Office of Naval Research and WRAIR. WRAIR is but a part of a multi-center study to provide data for the eventual licensure of this experimental system. Four other sites are performing *in vitro* evaluations similar to the protocol #714 and one other site is performing red cell survival studies on Haemonetics 215 processed red cells, similar to protocol #715. All testing except the 24 hour post-transfusion red cell survival measurements, however was performed at the Naval Blood Research Laboratory (NBRL) in Boston, MA.

Locally, volunteers were recruited, blood drawn and processed with the Haemonetics instrument, stored either in the frozen or liquid state for the prescribed time, and samples collected for shipment to NBRL.

Protocol #714 was completed and eight of eleven red cell survival studies for protocol #715 were completed at the conclusion of the report period.

BACKGROUND:

Both protocols are designed to evaluate an instrument developed by the Haemonetics Corporation, Braintree, MA, which automates both the glycerolization and deglycerolization processes required for frozen storage of red cells. But more important, the instrument is designed to perform all steps in a functionally "closed" system. Currently available systems instead are "open" systems. The distinction between "open" and closed" systems is critical because blood processed in open systems is subject to the FDA imposed restriction of a 24 hours post thawing and deglycerolization shelf life. This instrument is of value to the military blood program because a closed system, not available with current methods, would allow extension of the post-processing, liquid storage shelf life to two weeks, thereby greatly expanding the flexibility and utility of frozen blood. Furthermore, the degree of automation incorporated into the system will significantly minimize the technician intervention and manipulation required by the current processes and create a more uniform, standardized product.

METHODS:

WRAIR #714:

Twenty-one volunteers each donated 450 mL of blood which was collected into CPDA-1 (Fenwal Code 4R-1243P Roundlake, IL) All volunteers provided their informed consent and meet all criteria for whole blood donation for transfusion as specified by the American Association of Blood Banks and the FDA.^{14,15} In addition, volunteers were excluded if their blood contained sickle hemoglobin. The whole blood was centrifuged and sufficient plasma removed to produce an initial storage hematocrit of approximately 75%. The blood was stored for six days at 4°C. On the sixth day after collection, the red cells were glycerolized according to the instrument protocol and the glycerolized blood frozen at -80°C for at least 14 days. The frozen unit was

then thawed at 37°C and deglycerolized using the instrument's programmed protocol. The final stage of the deglycerolization protocol is the addition of AS-3. The deglycerolized cells were stored again at 4°C, this time for 14 days. Samples were collected from the unit at each stage of processing beginning with the freshly collected whole blood and ending with the final product after it was stored for 14 days. In addition, samples were collected from the supernatant wash at the conclusion of the deglycerolization step. All samples were sent to the NBRL for testing.

WRAIR #715:

All processes and procedures were repeated for the collection and processing of the blood from the eleven volunteers who consented for *in vivo* portion of the evaluation. Between the tenth and thirteenth day of liquid storage, the stored blood was inspected and cultured for bacterial contamination. At the conclusion of the final 14 day 4°C storage period, the volunteer returned to the laboratory for the *in vivo* recovery and survival study using the dual label method described by Moroff and colleagues.¹⁶

RESULTS: (preliminary)

Preliminary data analysis indicates the red cell recovery exceeded the minimum of 85%, i.e., greater than 85% of the red cells frozen were recovered in the final product bag and would thus be available for transfusion. Twenty-four hour post transfusion red cell survival averaged 82% for the first six volunteers who had completed survival studies; none were less than 77%. The gathering of data from the other participating sites is on going and analysis of either our data or the composite data is pending.

DISCUSSION / CONCLUSION: (preliminary)

The instrument performance meets or exceeds the industry standard of at least 85% recovery of red cells from the initiation of the freezing process to conclusion. Of the cells recovered, preliminary results indicate a more than adequate 24-hour post transfusion survival. Subjectively evaluated, the instrument was relatively easy to use and required far less operator intervention and attention than currently licensed instruments. Initial evaluation would indicate instrument is performing as expected and would be of value to the military blood program.

I. *In vitro* comparison of Red Blood Cells Stored in EAS-61 in Polyolefin versus Polyvinyl Chloride Storage Bags.

STATUS:

The data gathering and data analysis is complete. A manuscript for publication in a scientific journal is being prepared.

BACKGROUND:

The impetus for this study was threefold. First, the polyvinyl chloride (PVC) plastic used in blood collection system bags, as well as most of the IV solution bags and dialysis equipment, retains its pliability through the use of a plasticizer diethylhexylphthalate (DEHP). DEHP is a known health hazard in laboratory animals and a perceived health risk in humans. Second, the manufacture of PVC plastic requires the use of vinyl chloride, a carcinogen. Third, the incineration of PVC, the customary method of medical waste disposal, produces a variable amount of dioxins, another health and environmental hazard. For these reasons, it has been proposed to replace PVC with other non-PVC plastics. There is some evidence in the literature, however, that DEHP has a beneficial effect on red cell storage,^{17,18,19,20,21} and the storage lesion, i.e., the deterioration of the red cells as measured by loss of ATP, disk to sphere morphological changes and hemolysis, is accelerated when red cells are stored in non-PVC containers. This study was designed to determine the extent of the detriment created by storage in polyolefin plastic (PO), a non-PVC plastic, and to determine if the superior red cell preservation qualities of the experimental additive solution EAS-61 was sufficient to overcome the deficit created by depriving the red cells of exposure to DEHP during storage.

METHODS:

Volunteer donors, who had given their consent for collection of a unit of blood for another protocol, the harvesting of their anthrax-immune plasma, who gave their additional consent for the use of the red cells from their donation for use in other research, and who met the criteria for whole blood donation for transfusion,^{14,15} donated 450 mL of blood. The blood was collected into a specially prepared 1.0 L PO collection bag (Baxter Healthcare, 4R2238, Roundlake, IL) by

transferring 63 mL of anticoagulant preservative CPB from the primary collection bag of a commercially available collection system. In addition, a phlebotomy needle and a short segment of tubing were also added to the PO collection bag. All tubing connections were made with a sterile connecting device (SCD 312, Terumo Medical, Elkton, MD). The CPD and collection needle with tubing was removed from a commercially available blood collection system (Terumo Medical, BB*AGD456A, Elkton, MD). Twenty-four units were pooled in groups of four; each group of four units was ABO identical and pooled into a PO bag (Baxter Healthcare, 4R2023, Roundlake, IL). Each pool was divided into four equal aliquots, two into PVC bags (Baxter Healthcare, 4R2238, Roundlake, IL) and two into PO bags of the same composition as the collection and pooling bags. Into one of each set, 100 mL of EAS-61 was added; to the other, 200 mL was added. The EAS-61, 26 mM NaCl, 2 mM adenine, 50mM glucose, 20 mM mannitol, 12mM dibasic sodium phosphate, was laboratory prepared from high purity reagents and chemicals and tested for sterility and pyrogenicity prior to use. All bags were sampled for analysis of a variety of chemical and morphologic parameters weekly during the eleven weeks of 4°C refrigerated storage. Comparison of means of measured values at given times between the arms of the trial were evaluated by analysis of variance. Probabilities less likely than 0.05 were considered statistically significant.

RESULTS:

Significant differences were observed between the PVC and PO stored cells in fractional hemolysis, RBC morphology and ATP concentrations. Between the third and fourth weeks of storage, the hemolysis in the PO stored cells exceeded 1%, the maximum acceptable for FDA licensure as a blood storage system. Not until between the eighth and ninth week did the hemolysis exceed the 1% limit in the PVC store red cells. Three weeks into the study, it became apparent that the morphological appearances of all red cell samples were substantially worse in this study when compared to historical controls of EAS-61 collected into and stored in PVC. Morphological appearance continued to deteriorate until the final assessments were made. During the first two to three weeks of storage, the ATP content of the cells stored in PVC was remarkably higher, in either 100 mL or 200 mL of storage solution. However, by seven weeks of storage, only the cells stored in PVC and 200 mL of storage solution retained an advantage in

higher ATP content. Volume of storage solution appears to have been the major determinant of glucose concentration, with cells stored in 200 mL of solution being dramatically higher than when 100 mL throughout storage. Supernatant pH was nearly identical under all storage condition.

DISCUSSION / CONCLUSION:

Red cells stored in EAS-61 in PVC had markedly less hemolysis and higher ATP concentrations than those stored in PO. Based on this limited *in vitro* study, the shelf-life of blood collected into and stored in PO plastic would be very limited. Hemolysis would limit packed red blood cell storage to 4 weeks in PO bags. Furthermore, the superior performance of EAS-61, compared to the currently licensed additive solutions when blood is stored in PVC, was insufficient to overcome the deficit of collection and storage in PO.

J. "Photochemical treatment of platelet concentrates infected with *Ehrlichia chaffeensis*", WRAIR #731, STATUS:

The research with psoralen sterilization of blood products, a minimal risk protocol, received IRB approval and was completed. This research was performed by Capt Miriam Montes, BSC, USAF, a student in the triservice Blood Bank Fellowship, under the direction of Dr. Lloyd Lippert. Collaborating in the research were LTC Daryl Kelly and CPT Joseph Temenak, from the Department of Rickettsial Diseases of the Naval Medical Research Center. Capt Montes was in the process of writing a Master's thesis describing this research as the quarter closed.

BACKGROUND:

This research evaluated the ability of psoralens to kill the intracellular pathogen *Ehrlichia chaffeensis* in deliberately infected platelet concentrates. *Ehrlichia chaffeensis* is the causative agent for one variant of the disease ehrlichiosis. This work is particularly timely given the current interests of the transfusion services community and the CDC in rickettsial organisms as possible emerging pathogens in the nation's blood supply. *E. chaffeensis* was first isolated from an army recruit stationed at Fort Chaffee, Arkansas.²² Several years later, in June 1997, several National

Guard members became ill after returning from their 2 week annual field training exercises at Fort Chaffee. Some of the soldiers reported becoming ill shortly after donating blood, and in response to these reports, a voluntary recall of blood components collected at the base during three separate blood drives was issued.²³ In the follow-up of these donations, transfusion-associated illness from a tick-borne pathogen was not demonstrated.²⁴ Although the pathogenesis of the human ehrlichiosis is not well understood, it is likely that asymptomatic infected individuals, with high titers of ehrlichiae in their blood, can potentially infect others if their blood is transfused. Psoralen-mediated photochemical inactivation has also been shown to inactivate a wide range of pathogens.

Psoralens reversibly intercalate between the bases of nucleic acids and under illumination from long-wave ultraviolet light (UVA)(300-400nm) form covalent linkages to pyrimidines in either DNA or RNA. Since these reactions may occur at either or both ends of the psoralen molecule, a covalent bridge referred to as a di-adduct may be formed between pyrimidines in opposite strands of double stranded nucleic acids but may also form in single stranded nucleic acid viruses. Both di-adducts and mono-adducts block nucleic acid replication and transcription.^{25,26}

This work is a continuation of a previous study in which we deliberately infected platelet concentrates with the scrub typhus agent, *Orientia tsutsugamushi*.²⁷ The scrub typhus agent was very sensitive to psoralen sterilization; in this study we are asking the question if at least one of the causative agents of ehrlichiosis is similarly sensitive. Complete details of this protocol were included in a previous report.

METHODS:

The organism selected for this study was *E. chaffeensis*, human monocytic strain (HME). All work with this organism was conducted in accordance with Center for Disease Control and Prevention guidelines for biosafety level 3 facilities. The study was carried out using platelet concentrates (PCs) consisting of a pool of four ABO-identical random platelet units and was performed in two parts. The first part, Trial 1, consisted of testing *E. chaffeensis* infected Vero cells (CCL81; African green monkey hypodiploid fibroblasts, Integrated Diagnostics, Baltimore, MD) being a proven cell line in which *E. chaffeensis* can be cultured and propagated. The second part, Trials 2 and 3, consisted of using *E. chaffeensis* in human mononuclear cells, a system more

closely approximating the clinical environment. Since *E. chaffeensis* is an obligate intracellular organism that primarily infects monocytes, human mononuclear cells isolated from 75 mL of whole blood were harvested and cells were infected with *E. chaffeensis* and hereafter seeded into the platelet concentrate (PC). Different amounts of *E. chaffeensis* infected Vero were used in Trails 2 versus Trail 3. The platelet concentrates were treated with concentrations of 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT), provided by the Cerus Corporation (Concord, CA), ranging from 0.20 mg/mL to 40 mg/mL combined with 12 min of UVA light of 5 J per ² cm. After treatment, the samples were immediately subcultured in Vero cells and examined for the presence of *Ehrlichia* using Giemsa staining and direct fluorescent antibody (DFA) staining. An additional sample for polymerase chain reaction (PCR) testing for evidence of ehrlichial DNA was also collected.

Table 4. Inoculation of platelet concentrate

Trial #	Type of infected cells used	% infected cells	Amount of inoculum (mL)	<i>E. chaffeensis</i> per mL of PC
First	Vero	63	0.8	4.4×10^4
Second	mononuclear	10	0.6	1.14×10^3
Third	mononuclear	10	1.0	7.3×10^3

RESULTS:

All testing indicated *E. chaffeensis* in PC was not inactivated by treatment with AMT in concentrations up to 40 µg/mL combined with 5 J/cm² UVA. Viable organisms were cultured from all completely treated samples, without regard to the source of the inoculum, infected Vero cells or infected human mononuclear cells. Though there was a trend indicating some degree of inactivation such as fewer organisms per infected cell or less prominent bands following electrophoresis of the PCR product, data not shown, none of the methods employed were quantitative. DFA and PCR testing indicated the cultured organisms were *E. chaffeensis*.

DISCUSSION / CONCLUSION:

Geimsa and direct fluorescent antibody (DFA) staining of culture cells and polymerase chain reaction (PCR) analysis of culture material all indicate no more than minimal effectiveness in reducing the number of organisms in all samples even with the highest concentrations of AMT tested. All testing indicated the *Ehrlichia chaffeensis* is far more resistant to sterilization with the psoralen AMT than the scrub typhus agent, *Orientia tsutsugamushi*. The scrub typhus agent was completely inactivated and killed by concentrations of AMT 200 fold less.²⁷

Cumulatively, these observations suggest *E. chaffeensis* is refractory to treatment with AMT and UVA illumination. Furthermore, sterilization of PC with AMT and UVA is selectively effective against arthropod-borne diseases which might be transmitted in blood.

K. "Evaluation of the in vivo viability of red blood cells after 8- and 9 -week storage in Terumo's EAS-61 Additive solution" WRAIR #713, Log #A-8655.

STATUS:

WRAIR #713 protocol outlines our plans to test an experimental solution in a minimum of ten volunteers after both eight weeks and nine weeks of storage in a random double crossover clinical trial using AS-5 as the control storage system. The EAS-61 additive solution is an investigational product developed at another institution under an extramural contract and to be manufactured under license to the Terumo Corporation. Survival of red cells stored in EAS-61 for nine weeks has exceeded the 75% minimum by a clear margin in limited testing performed in the contractor's laboratory. The data collected in this protocol will be used to support FDA licensure. No progress has occurred because the product to be tested is not available at this time.

GENERAL AND ADMINISTRATIVE

There have been no significant administrative changes in contract operation since the previous annual report. Instead, existing systems and processes for documentation, equipment maintenance and repair, and laboratory testing procedure validation have been maintained to meet research requirements.

Manuscripts of research results from contract supported research have been submitted for publication with some published during the report period. The status of each manuscript submission is detailed in previous sections of this report.

A significant amount of effort went into the relocation to the new WRAIR facility, Building 503 on the Forest Glen Campus of Walter Reed. All laboratory operations were suspended during the last two weeks of March 1999. The last of the equipment arrived in the new location on 30 March. Laboratory function was gradually reestablished during the following two months. The establishment of full laboratory function was delayed by two issues. First, the simultaneous implementation of a cradle-to-grave system for accountability of hazardous materials. In order to establish full accountability before the items were moved to the new location, the people responsible for implementing the system held our items, some up to six weeks, in order to complete registration and tagging of all items. The second delay was the result of damage to the multi-channel analyzer by the movers. Because the individuals responsible for payment of damages were untimely in their response, the repairs were not completed until nearly two months after the analyzer was moved. Because of space limitations in the new facility and the reduced need for redundancy of critical equipment, significant amounts of excess equipment were turned-in for disposal through government channels. The government also had the opportunity to replace a few pieces of outdated and bulky, cumbersome and oversized equipment with modern and more compact items. The resulting changes in the list of government furnished equipment was formalized as a contract modification.

SUMMARY

The contract staff has supported the BRD by operating and maintaining the Blood Storage Laboratory to support both existing and new requirements. Staff are trained and systems are in place which supported specific red cell survival protocols. The contract staff successfully operated the Laboratory in two locations. At the beginning of the FY the laboratory was located in leased space at 1413 Research Boulevard, Rockville, Maryland. In March of 1999, the Laboratory as relocated to the new WRAIR facility on the Forest Glen campus of the Walter Reed Army Medical Center, Silver Spring, Maryland and successfully reestablished. The BRD mission has been supported.

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Appendix 1

Contract Employees as of end of Fiscal Year 1999:

Lloyd E. Lippert, Ph.D., SBB(ASCP), Project Manager (Full-time)

Cynthia Oliver, B.S., MT(ASCP) SBB, Technologist (Full-time)

Heather Hill, B.S., Technologist (Full-Time)

Patricia Cowan, BSN, RN, Registered Nurse (Part-time)

Nicole Putnam-Frenchik, Administrative Assistant (Part-time)

The viability of autologous human red cells stored in additive solution 5 and exposed to 25°C for 24 hours

T.J. Reid, J.G. Babcock, C.P. Derse-Anthony, H.R. Hill, L.E. Lippert, and J.R. Hess

BACKGROUND: No data exist on the viability of red cells (RBCs) stored in modern additive solution systems and allowed to warm above 10°C.

STUDY DESIGN AND METHODS: In a randomized crossover study, 3 units of blood were collected at least 8 weeks apart from 11 volunteer donors and stored in additive solution 5 (AS-5). Of 3 units from each volunteer, 1 was stored for 6 weeks at 4°C, 1 for 5 weeks at 4°C except for 24 hours at 25°C on Day 14, and 1 for 5 weeks at 4°C except for 24 hours at 25°C on Day 28. Units were sampled periodically during storage; at the end of storage, viability was measured by the $^{99m}\text{Tc}/^{51}\text{Cr}$ double-label method.

RESULTS: RBC viability was not significantly different in the storage protocols. Less than 1 percent of stored cells hemolyzed. RBC ATP concentrations at the end of storage correlated with viability and were approximately equal in the warmed units after 30 days' storage and the conventionally stored units after 42 days.

CONCLUSIONS: The data suggest that RBCs stored in AS-5 and allowed to warm to 25°C for 24 hours lose about 12 days of their shelf life.

Red cells (RBCs) are normally stored in the refrigerator and shipped on wet ice.¹ This practice maintains the viability of transfused RBCs and limits bacterial growth. It has been codified in Food and Drug Administration (FDA) regulations,² which state that RBCs may not be issued unless they have been stored at temperatures between 1 and 6°C and shipped at temperatures between 1 and 10°C.

Two groups have examined the viability of RBCs stored at room temperature. In 1947, Gibson and colleagues³ described extensive studies of the effects of warming RBCs during storage. Donors were given radioactive iron, the radioactive cells were transfused to volunteer recipients, and the recovery of cells at 24 hours was measured. In two of these cases, blood was stored for 24 hours at 20 to 25°C. These units of whole blood were drawn into ACD and Alsever's solution, respectively, stored for 46 hours at 4°C, allowed to stand at room temperature (20-25°C) for 24 hours, and returned to storage at 4°C until their transfusion 18 days after being drawn. The recovery fraction was approximately 72 percent in the ACD-stored unit and about

ABBREVIATIONS: AS-5 = additive solution 5; FDA = Food and Drug Administration; Hb = hemoglobin; RBC(s) = red cell(s).

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30 percent with storage in Alsever's solution. The other study is that of Shields in 1970.⁴ Shields enrolled 72 volunteers, collected their blood in ACD, and stored it at 4°C except that one-half of the units were warmed to 22°C for the 24 hours preceding their transfusion. One-third of the units were returned at each of 7, 21, and 28 days after drawing, and recovery was measured with the ³²P/⁵¹Cr double-isotope method. Twenty-four hours of room-temperature storage significantly reduced viability, by an amount approximately equal to the loss of viability seen with an additional week of 1 to 6°C storage. Both Gibson and colleagues and Shields concluded that warm storage reduced viability, but Shields suggested that periods of warming up to 24 hours might be tolerated.

There are no data available on the viability of RBCs stored at room temperature in modern additive solutions.⁵ Such evidence might support changes in the regulations regarding storage, such as the recent extension of the room-temperature holding time before the separation of platelets.^{6,7} Performance-based standards and regulations that allowed additional storage time outside the range of 1 to 6°C are expected to improve the availability of blood in emergency rooms and surgical suites, while reducing the destruction of units issued but not transfused. Such data may also allow medical directors to make evidence-based decisions about the usability of RBCs after nonstandard storage in emergencies.

To test Shields's rule of thumb, that a day at room temperature reduces RBC viability by a week, we compared the viability of RBCs stored in additive solution 5 (AS-5) at 1 to 6°C for 6 weeks to that of RBCs stored for 5 weeks when cells were warmed to 25°C for 24 hours on Day 14 or Day 28. We performed these steps in a randomized crossover study.

MATERIALS AND METHODS

Volunteers

Fifteen healthy volunteers meeting standard blood donor criteria¹ were enrolled after giving informed consent in accordance with a protocol approved by the Institutional Review Board of Walter Reed Army Institute of Research and the U.S. Army's Human Subjects Research Review Board. Volunteers were tested to exclude sickle hemoglobin trait (Sicklescreen, Pacific Hemostasis, Huntersville, NC) and abnormal osmotic fragility (Unopette Test 5830, Becton Dickinson, Rutherford, NJ).

Study design

The study was conducted by using a crossover design. Each volunteer donated 3 units of blood with at least 8 weeks separating the donations. The 3 units were assigned randomly to storage in the following manner: 1 unit was stored for 6 weeks at 4°C (control unit), a second for 5 weeks at 4°C except for 24 hours at 25°C on Day 14, and a third for 5 weeks at 4°C except for 24 hours at 25°C on Day 28. The primary

outcome measurement was the viability, measured as the fractional *in vivo* recovery of the stored RBCs 24 hours after their return to the donor. We also measured postrecovery RBC survival and the changes in supernatant pH, whole-blood glucose concentration, RBC ATP concentration, and supernatant hemoglobin (Hb).

Blood unit preparation

Four hundred fifty mL ($\pm 10\%$) of blood was collected into CPD in the primary bag of an AS-5 triple-bag system (Optisol, Terumo Medical Corp., Somerset, NJ). Packed cells were prepared by centrifugation at $5000 \times g$ for 5 minutes at room temperature, and this was followed by the removal of plasma and the addition of the AS-5 to achieve a target storage hematocrit of approximately 60 percent. All units were sampled for *in vitro* testing and placed in refrigerated storage (1–6°C) within 2 hours of collection. Test units were stored for 5 weeks and control units for 6 weeks.

Warming

On the scheduled day of warming, test units were removed from the monitored blood bank refrigerator, sampled as described below, and placed in a 25°C incubator for 24 hours. At the end of the warming period, the units were removed, sampled again, and returned to the blood bank refrigerator.

In vitro measurements

Samples from stored units were collected into a small pouch attached to the residual donor needle tubing by using a sterile connecting device (SCD 312, Terumo). The battery of *in vitro* tests described below was performed on all units on Day 0 after the addition of AS-5; on Days 13, 14, 21, 27, and 28; and at the end of storage (Day 35 for test units and Day 42 for control units).

Blood pH was measured on stored blood supernatant by using a clinical blood gas analyzer (Model 855, CIBA-Corning, Medfield, MA). Total Hb concentration and RBC counts were measured on stored blood with a clinical hematology analyzer (Hematology Cell Counter System Series 9110+, Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically by using the modified Drabkin assay.⁸ The percentage of hemolysis was determined by the ratio of supernatant Hb to total Hb. Centrifuged microhematocrits (Clay Adams, Becton Dickinson, Rutherford, NJ) were performed to ascertain stored-unit volume fractions.

Whole-blood glucose and RBC ATP concentrations were measured in supernatants of deproteinized whole blood. Whole-blood aliquots were mixed with cold, 10-percent trichloroacetic acid to precipitate blood proteins and centrifuged at $2700 \times g$ for 10 minutes, and the protein-free supernatant was frozen at –80°C until it was tested. Glucose concentrations were determined with a clinical chemistry

analyzer (Cobas Fara, Roche Diagnostic Systems, Nutley, NJ). Glucose consumption was determined from changes in whole-blood glucose concentrations corrected for RBC volume fraction. ATP was assayed enzymatically by using commercially available test kits (Procedure 366-UV, Sigma Diagnostics, St. Louis, MO). In studies in our laboratory, 10- and 12-percent trichloroacetic acid precipitates gave equivalent RBC ATP concentrations (data not shown).

Check for bacterial contamination

Three to 4 days before the end of storage, aliquots from each unit were tested for bacterial contamination in broth and agar cultures by using a commercial blood culture system (Septi-Chek Blood Culture Bottle and Slide, Becton Dickinson Microbiology Systems, Cockeysville, MD). Absence of growth in the cultures was documented before the unit was sampled for return.

In vivo RBC recovery and survival measurement

After 5 or 6 weeks of storage, in vivo RBC recovery was measured 24 hours after autologous return by using both single- and double-radioisotope procedures.^{9,10} In brief, a sample of the stored blood was labeled with ⁵¹Cr. Concurrently, a fresh blood sample was collected from the volunteer and labeled with ^{99m}Tc. Carefully measured aliquots of the radiolabeled RBCs were mixed and rapidly returned. Blood samples were collected 5, 7.5, 10, 12.5, 15, 20, and 30 minutes after the return and again at 24 hours, 7 days, and 14 days after return. Radioactivity of the samples was measured in a gamma counter (Model 1480, Wallac, Turku, Finland), except for samples from two volunteers, in which the ^{99m}Tc counts were performed in a different counter (CLINGAMMA Model 1272, Wallac). Gamma emissions from ^{99m}Tc-radiolabeled cells were measured in the samples collected during the 30 minutes after return and used to determine the RBC volume at the time of return. The activity from ⁵¹Cr-labeled cells was measured on all samples and used to calculate a second RBC volume and the recovery and survival of the returned RBCs. Survival was calculated as the fraction of cells recovered at 24 hours that were still in circulation at 7 and 14 days by using the 1-percent-per-day estimate of ⁵¹Cr elution from RBCs.¹¹

Statistical analysis

One-way ANOVA was used to examine the relationships between outcomes and mode of storage as well as the differences in measures among the donors. Pearson correlation analysis was used to examine the ef-

fect of end-of-storage RBC ATP concentration on these relationships. These calculations were performed with software (Systat 6.1, SPSS, Chicago, IL). Calculations of group means and their standard errors were performed with software (Excel for Windows 97, Microsoft Corp., Redmond, WA). A probability of less than 0.05 was considered significant.

RESULTS

Volunteers

Eleven of 15 enrolled volunteers completed the study. They are described in Table 1. Of the volunteers who withdrew, one was removed from the study because of a persistently low hematocrit after the first donation, one moved from the metropolitan area, and two withdrew for personal reasons.

Uniformity of RBC unit preparation

Storage hematocrit of the AS-5 units varied from 55 to 68 percent, averaging 62 ± 1 percent (mean \pm SEM). The mean hematocrits of the stored units were not different among the storage categories or the volunteers.

In vitro measures of RBC metabolism and breakdown during storage

Whole-blood glucose, pH, RBC ATP concentrations, and free Hb were measured at specified times in the course of storage. The whole-blood glucose concentration decreased at an average rate of 7.4 mg per dL per day during storage at 4°C (Fig. 1A). This rate corresponds to 0.028 μ M glucose per mL of RBCs per hour. Warming the cells to 25°C increased the rate of decrease of whole-blood glucose concentration 12-fold, to 86 mg per dL per day. The pH also decreased and in a manner similar to that of glucose concentration (Fig. 1B). RBC ATP concentrations decreased

TABLE 1. Characteristics of volunteers who completed the study

Number	Age	Sex	Hematocrit		Diseases, drugs, or other abnormality
			Donor*	In units†	
1	31	M	40/42/41	57/65/62	Loratadine/pseudoephedrine
2	44	M	47/47/45	64/63/68	
3	32	M	40/42/41	62/60/60	Propranolol, fluoxetine
4	33	M	43/40/43	62/68/63	Type II diabetes, no treatment
6	30	M	41/40/36	57/62/60	Treated alcoholism
10	27	M	39/40/40	62/62/62	
11	53	M	42/43/41	62/61/58	
12	27	M	41/39/43	57/62/65	
13	41	F	40/40/40	64/65/64	
14	48	M	41/40/41	62/62/66	
15	44	M	48/46/49	58/55/62	

* Hematocrit at donation of the unit to be stored at 4°C for 5 weeks with warming on Day 14/of the unit to be stored at 4°C for 5 weeks with warming on Day 28/of the unit stored at 4°C for 6 weeks (control unit).

† Hematocrit of the unit stored at 4°C for 5 weeks with warming on Day 14/hematocrit of the unit stored at 4°C for 5 weeks with warming on Day 28/hematocrit of the unit stored at 4°C for 6 weeks (control unit).

more rapidly in the days after warming (Fig. 1C). Mean RBC ATP concentrations in the warmed cells at 30 days of storage were approximately equal to those of the convention-

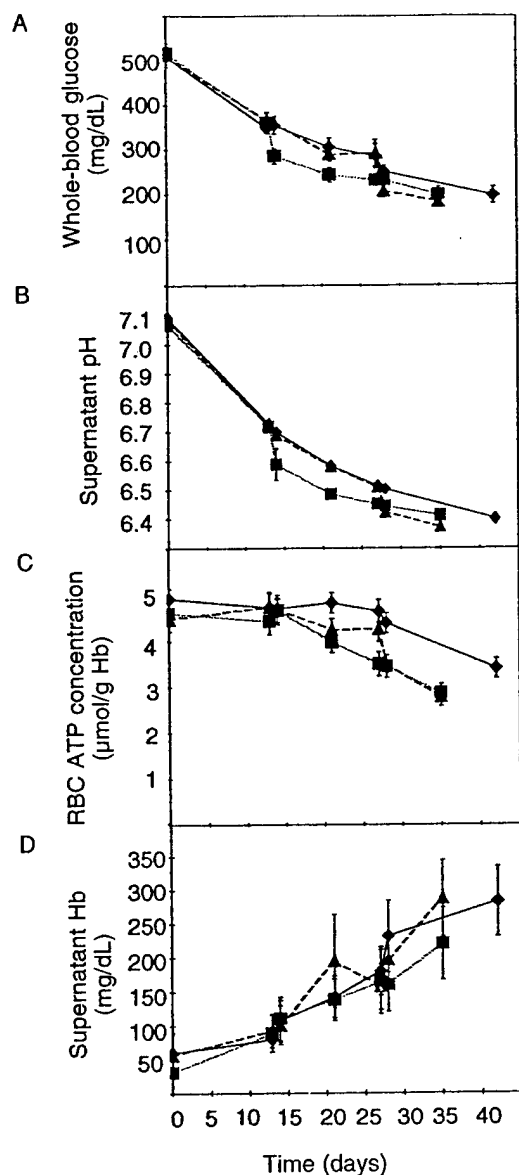


Fig. 1. Measures (mean \pm SEM, $n = 11$ at all points) of A) whole-blood glucose concentration, B) supernatant pH, C) RBC ATP concentration, and D) supernatant Hb concentration. The three arms of the study are 1) 4°C storage for 6 weeks ($-\diamond-$), 2) 4°C storage for 5 weeks with warming to 25°C on Day 14 ($---\blacksquare---$), and 3) 4°C storage for 5 weeks with warming to 25°C on Day 28 ($-\blacktriangle-$). The decrease in pH corresponds to the consumption of glucose, and both are accelerated by warming. RBC ATP concentration decreases more rapidly after warming, presumably because ATP synthesis is slower at reduced pH. RBC lysis measured as supernatant Hb concentration increases with storage time but never reaches a value of 520 mg per dL, equivalent to lysis of 1 percent of the cells.

ally stored cells after 42 days. RBC ATP concentrations at the end of storage, 3.4 ± 0.2 , 2.9 ± 0.2 , and 2.8 ± 0.2 μmol per g of Hb in the control units, the test units warmed on Day 14, and the test units warmed on Day 28, respectively, were not significantly lower in the warmed units ($p = 0.077$), but varied in a manner that was highly donor-specific (Fig. 2A, $p = 0.007$). RBC ATP concentrations at the beginning of storage did not predict concentrations at the end of storage.

Lack of bacterial contamination

Three to 4 days before the end of the assigned storage period, all units were cultured for bacteria; none showed evi-

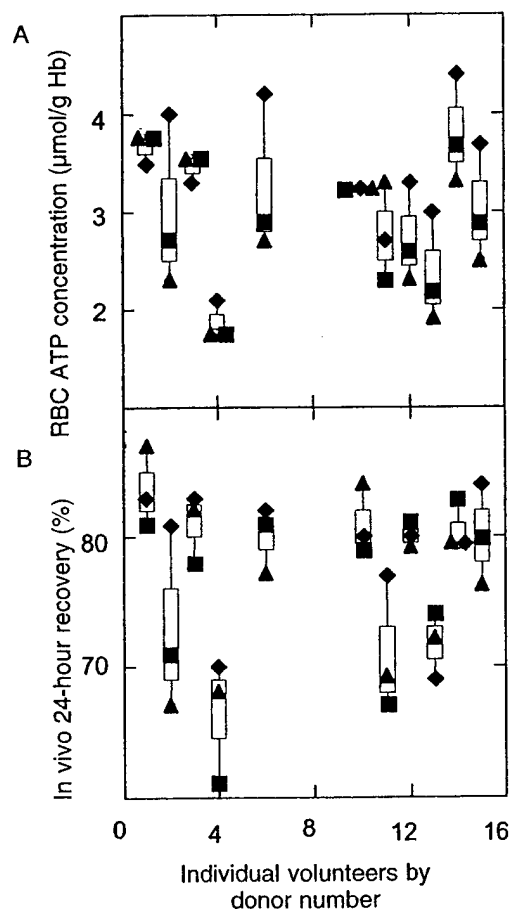


Fig. 2. Individual measurements of A) RBC ATP concentration at the end of storage and B) single-label 24-hour RBC recovery in the individual donors. The three arms of the study are 1) 4°C storage for 6 weeks (\diamond), 2) 4°C storage for 5 weeks with warming to 25°C on Day 14 (\blacksquare), and 3) 4°C storage for 5 weeks with warming to 25°C on Day 28 (\blacktriangle). RBC ATP concentration at the end of storage is correlated with viability, but it has little discriminant value for choosing units that store well. The poor survival of RBCs from a few donors represents the greatest source of variability in this study. The variability in measurements of RBC volume with $^{99\text{m}}\text{Tc}$ in the same donor make the double-label survival measurements less precise.

dence of contamination or growth. At the end of the assigned storage period, the mean fraction of hemolyzed RBCs was less than 1 percent under all conditions of storage (Fig. 1D).

RBC recovery

The measured recoveries of RBCs returned to original donors at the end of storage were not statistically different in the three storage groups. This was true whether recovery was measured by the ^{51}Cr single-label method or by the $^{99\text{m}}\text{Tc}/^{51}\text{Cr}$ double-label method (Fig. 3). The values for the single-label method were 79 ± 2 percent for cells stored at 4°C for 6 weeks, 76 ± 2 percent for cells warmed on Day 14, and 76 ± 2 percent for cells warmed on Day 28. The double-label method gave values that averaged 2.5 percent lower. The mean values were 78 ± 3 percent, 75 ± 2 percent, and 72 ± 2 percent for the cells stored at 4°C for 6 weeks, for the cells warmed on Day 14, and for the cells warmed on Day 28, respectively. Although the recovery percentages of the warmed RBCs are lower on average, this difference was not significant because of the large variability of recoveries under all conditions among the individual volunteers. This difference among donors is best seen in the single-label measurements (Fig. 2B, $p = 0.003$). The double-label recov-

ery measurements were performed to correct for the systematic error caused by the early and unmeasured loss of ^{51}Cr -labeled stored cells in the first 5 minutes after return but before the start of mixing and sampling. The three measures of $^{99\text{m}}\text{Tc}$ RBC mass in the individual volunteers were reproducible to 5 ± 3 percent with a range of 1 to 10 percent. Thus, the double-label recovery measurements correct a 2.5-percent systematic error, but in doing so, they induce an additional 5-percent random error because of the inherent stochastic errors of radiation counting and microhematocrit measurement. For analysis within a group, the greater precision of the individual single-label measures brings out correlations. For the comparison of average viability, the means of groups measured with the double-label method are more accurate.

RBC survival

The viable mass of returned RBCs that were circulating 24 hours after the return declined at about 1.4 percent per day during the next 2 weeks in all volunteers and under all conditions of storage. At 7 days, the fractional survival averaged 89 ± 1 percent; at 14 days, it was 81 ± 1 percent.

Correlation between RBC ATP concentration and RBC recovery

The RBC recovery measured by either the single- or double-label method correlated with the RBC ATP concentration with the correlation coefficient $n = 0.627$ ($p = 0.001$) for the single-label method and $r = 0.496$ ($p = 0.003$) for the double-label method. The fraction of the total variability in RBC recovery explained by the correlation with ATP concentration (the coefficient of variation [r^2]) was 0.393 and 0.246 for the single- and double-label methods, respectively. Because the range of errors induced by the double-label method was almost as large as the variability in measured recovery, it reduced r^2 by almost one-half.

The relationship between the slope of the regression line for RBC recovery on RBC ATP concentration was an 8-percent decrease in viability (RBC recovery) with each μmol decrease per g of Hb in RBC ATP concentration. The lower RBC ATP concentration observed in the warmed units at the end of storage ($0.55 \mu\text{mol/g Hb}$) predicts almost exactly the 4.5-percent mean decrease in observed RBC recovery. The slope of the decrease in RBC ATP concentration in the last weeks of storage seen in Fig. 1C ($0.7 \mu\text{mol/g Hb/week}$) makes this difference equivalent to 5 fewer days of storage.

DISCUSSION

This study shows that RBCs stored in AS-5 and warmed to 25°C for 24 hours on either Day 14 or 28 in the course of 35 days of 4°C storage have an in vivo recovery and survival that are not statistically different from those of RBCs stored at 4°C for 42 days. Nor do they display excessive hemolysis.

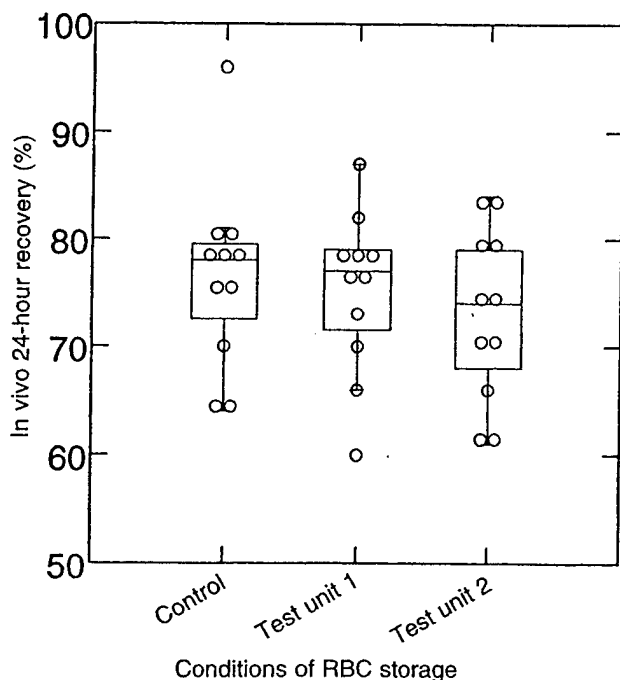


Fig. 3. Viability measured as the 24-hour in vivo RBC recovery using the $^{99\text{m}}\text{Tc}/^{51}\text{Cr}$ double-label method did not vary under the different conditions of storage. Control = units stored at 4°C for 6 weeks, test unit 1 = units stored at 4°C for 5 weeks with 25°C storage on Day 14, and test unit 2 = units stored at 4°C for 5 weeks with 25°C storage on Day 28.

The decrease in viability associated with warming seems to be caused by the temperature-related increased rate of metabolism and the resulting earlier onset of reduced pH and conditions that adversely affect the maintenance of RBC ATP concentrations. Three separate measures—the rates of glucose consumption, the times when RBC ATP concentrations are equal, and the correlation of rates of decline in RBC ATP concentrations with RBC recovery—converge to suggest that one day of storage at 25°C reduces the storage time by 12 days. Thus, RBCs transfused after refrigerated storage for 29 days and one day at 25°C are expected to display the required 75-percent mean viability with less than 1-percent hemolysis.¹⁰ Further, because the decrease in RBC ATP concentrations in the later weeks of RBC storage is linear with time, a 2-hour exposure at 25°C probably shortens the time to unacceptable RBC recovery by a day. However, as Shields⁴ noted, such short exposures to higher temperatures produce differences in RBC recovery that are too small to measure.

It seems likely that the effects of temperature on RBC recovery that were measured here during storage in AS-5 also apply to the other additive solutions with the same volumes and similar composition: saline-adenine-glucose-mannitol and AS-1 (Adsol, Baxter Healthcare, Roundlake, IL). We previously reported measures of RBC ATP concentrations after normal and warm storage of RBCs in CPDA-1 and showed a quantitatively similar decrease.¹² AS-3 (Nutricel, Medsep Corp., Covina, CA) shows a similar decrease in RBC ATP concentrations with time in the cold, but no data exist on its performance with warming.

According to the first edition of what is now called the *Technical Manual*, "When blood is issued for transfusion, it must be given to the patient within a reasonable period of time or promptly returned to the blood bank. If the blood has been beyond the direct control of the blood bank personnel for 24 hours or more, no responsibility can be taken for its safety or quality..."^{13(p 71)} This statement reflected a general understanding of the temperature-dependent rates of RBC metabolism and bacteria growth. Now, the standards¹ of the American Association of Blood Banks and FDA regulations state that RBCs may not be issued unless they have been stored at temperatures between 1 and 6°C and shipped at temperatures between 1 and 10°C. The regulation has been widely interpreted to mean that RBCs that have spent more than 30 minutes out of the refrigerator, and therefore might have warmed above 10°C, should be destroyed.¹⁴ The actual data on which this "30-minute rule" was based showed that units of whole blood in plastic bags warmed to temperatures above 10°C in 45 to 60 minutes after removal from blood bank refrigerators.¹⁵ In the ninth edition of the *Technical Manual*, the justification for the rule was stated: "Warming the blood beyond... (10°C), even with subsequent cooling, tends to accelerate red cell metabolism, produce hemolysis, and may permit bacterial growth

in the unit."^{16(p 49-50)} Bacteria growth in warmed units of RBCs is rare.¹⁷

The 30-minute rule is a measurable and enforceable quality control standard. However, it is wasteful of blood and often limits the availability of blood at sites of critical care. Surgeons rightfully complain. The 30-minute rule should be reconsidered in the light of present knowledge and should be dealt with explicitly in future FDA regulations and American Association of Blood Banks standards. In the past, the FDA has not allowed alternative procedures for RBC storage under 21 CFR 640.120 where the temperature of RBCs exceeded 10°C. In the future, a revised 30-minute rule should lead to an evidence-based decision regarding the safety and viability of the units in question, not to an automatic requirement for destruction. Such a decision should be made within a framework of revised government regulations, accrediting agency standards, and facility guidelines by a technologist or the medical director.

In the meantime, these data probably have use only for medical directors making critical decisions about the safety of units accidentally exposed to room temperatures in the course of emergency situations, when other blood of known safety and viability is not available. The present standards provide excellent protection for the viability and bacteriologic safety of RBCs and should not be violated except in true emergencies.

ACKNOWLEDGMENTS

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Appendix 3

THE EFFECTS OF PHOSPHATE, pH, AND ADDITIVE SOLUTION VOLUME ON RBCS STORED IN SALINE-ADENINE-GLUCOSE-MANNITOL ADDITIVE SOLUTIONS.

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Running Head: Effect of additive solution volume

Introduction:

Red blood cell (RBC) storage systems with longer outdating will have many beneficial effects.¹ The burden of non-viable RBCs will be reduced for most transfusion recipients. Some of the several hundred thousand RBCs that now outdate each year will not be lost. The ability to maintain adequate emergency stocks in remote locations will be enhanced. Autologous transfusion schemes will function better if RBCs can be stored longer, have better viability, and contain fewer breakdown products.

Developing better RBC storage systems has been slow. For the last decade, 6-week RBC storage has been the U.S. standard. That standard is based on the use of 100 mL additive solutions containing either saline, adenine, glucose, and mannitol (SAG-M, as in AS-1 and AS-5) or saline, adenine, glucose, and monosodium phosphate (SAG-P, as in AS-3).² Two 7-week RBC storage systems have been licensed in Europe, Erythrosol³ and PAGGS-M,⁴ however, the 24-hour viability of cells stored in these solutions is barely 75%. The promise of 14-week RBC storage promised in the 1986 publication of Meryman, Hornblower, and Syring⁵ has not been realized in transfusable solutions. Nevertheless, the recognition that longterm viability is possible in solutions that drive ATP synthesis, remains the most likely path for future development.

Here we report the results of three "pooling" studies that measured RBC ATP concentrations in the face of increasing volumes of the additive solutions. The experimental additive solutions (EASs) with increased phosphate, pH, and volume worked well, where as the traditional SAG-M solutions performed less well at greater dilutions. These studies were performed in two different laboratories. They are reported together because they help explain why present storage solutions limit viability to 6 weeks and how transfusable solutions might be

Structured Abstract:

Background: RBC ATP concentrations are the most important correlate of RBC viability. These studies tested whether increased additive solution volume, pH, and phosphate content increased stored RBC ATP concentrations.

Study Design and Methods: In three studies, units of blood were pooled in groups of 3 or 4 units and realiquoted as combined units to reduce intra-donor differences. Thirty units were stored in AS-1 at RBC concentrations equivalent to 100, 200, or 300 mL of additive solution. RBCs from 24 units were stored in 100, 200, 300, or 400 mL volumes of an experimental additive solution, EAS-61. Thirty units were stored in 100 mL of AS-5, 200 mL of EAS-61, or 300 mL of EAS-64. RBC ATP concentration and other measures of RBC metabolism and function were measured weekly.

Results: RBC ATP concentrations decreased sooner with storage in increasing volumes of AS-1. In EAS-61 and EAS-64, RBC ATP concentrations initially increased and stayed elevated longer with increasing additive solution volume.

Conclusions: Addition of disodium phosphate to saline-adenine-glucose-mannitol additive solutions increases the RBC ATP concentrations. Reducing storage hematocrit appears to have an separate beneficial effect in reducing hemolysis

Key Words: Blood storage, RBC storage, Humans, RBC ATP concentrations

formulated that will extend RBC viability beyond that limit.

Materials and Methods:

Volunteers

In two studies, 30 healthy volunteers, who were donating full units of blood for the collection of anthrax-immune plasma, consented to their RBCs being used for storage studies. The donors met standard blood donor criteria and gave informed consent in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research and the U.S. Army's Human Use Review Board. Volunteers were tested to exclude sickle hemoglobin trait (Sicklescreen®, Pacific Hemostasis, Huntersville, NC) and abnormal osmotic fragility (Unopette™ Test 5830, Becton Dickinson, Rutherford, NJ).

In another study, 24 volunteers donated full units of blood under protocols approved by the Hoxworth Blood Center and the University of Cincinnati's Institutional Review Board.

Storage Solutions

The compositions of AS-1 (ADSOL®, Baxter Healthcare Corp., Roundlake, IL), AS-5 (Optisol®, Terumo Medical Corp., Somerset, NJ), EAS-61 and EAS-64 are compared in Table 1. The significant differences in the solutions are the reduced salt content and the presence of disodium phosphate in the EASs. The presence of disodium phosphate raises the pH of the EASs to about 8.5 compared to 5.8 in AS-1 and AS-5.

The EASs were made in the laboratory from high purity adenine, sugars, and salts and sterilely filtered into one-liter storage bags. The bags were held at room temperature for two

weeks. The solutions were then cultured and the cultures incubated for another two weeks. When sterility was confirmed by the absence of bacterial growth for 14 days, the solutions were aliquoted by weight into 600 mL bags. All connections were made using a sterile connecting device (SCD 312, Terumo Medical Corp. Elkton, MD).

Study Design

We conducted three "pooling" studies to evaluate RBC metabolism and physiology over the course of storage. Pooling reduces the largest source of variability in conventional blood storage studies, the differences between the RBCs from different donors, by placing some of the cells from every donor in every arm of the study while maintaining conventional unit size and geometry. In each of the three studies the RBC units were grouped into sets of 3 or 4 ABO-matched units, each set was then pooled, mixed, and realiquoted into identical pooled units. For example, in the first study, thirty RBC units were collected and grouped into ten sets of three ABO-matched units. Each set of three matched units was then pooled, mixed, and realiquoted as three identical pooled units. Ten pooled units, one from each set, were used in each arm of the study.

In the first study units were stored in AS-1 at RBC concentrations equivalent to dilution in 100, 200, and 300 mL of additive solution. In the second study, units were stored in 100, 200, 300, or 400 mL volumes of the experimental additive solution EAS-61. In the third study, units were stored in 100 mL of AS-5, 200 mL of EAS-61 or 300 mL of EAS-64.

The problem with "pooling" is that after mixing, it is no longer possible to return the autologous cells to the original donor and measure viability. The studies can only be used to

measure metabolic and red cell physiologic end points. Thus, electrolytes, pH, metabolites, RBC morphology scores and RBC ATP concentrations were measured weekly during storage using sterile sampling techniques.

RBC Unit Preparation

First study, AS-1 units: Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CPD anticoagulant in the primary polyvinyl-chloride (PVC) bag of an AS-1 triple-bag collection set (code 4R-14-36, Baxter Healthcare Corporation, Deerfield, IL). Packed cells were prepared by centrifugation at $5,000 \times g$ for 5 minutes at room temperature followed by the removal of sufficient plasma to achieve a hematocrit of 75%. ABO-matched PRBCs were then pooled in groups of three in 1 L sterile bags (code 4R-20-32, Baxter Healthcare Corporation, Deerfield, IL), mixed thoroughly, and aliquoted into the study units by weight using sterile tubing connection for all the transfers. The study units were produced in 600 mL transfer bags (code 4R-20-23, Baxter Healthcare Corporation, Deerfield, IL) by mixing the PRBC aliquot with sufficient additive solution to produce final storage hematocrits of 55%, 43%, and 35%. The result is essentially equivalent to the addition of 100, 200, or 300 mL of additive solution to 200 mL of RBC respectively.

Second study, EAS-61 units: Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CPD anticoagulant in the primary polyvinyl-chloride (PVC) bag of an AS-1 triple-bag collection set (code 4R-14-36, Baxter Healthcare Corporation, Deerfield, IL). Packed cells were prepared by centrifugation at $5,000 \times g$ for 5 minutes at room temperature, followed by removal of plasma to achieve a target hematocrit of 80%. PRBCs were pooled, as

above, in sets of four and realiquoted. Finally, EAS-61 was added in amounts of 100, 200, 300, and 400 mL to achieve target storage hematocrits of about 66, 55, 46, and 40%. The storage hematocrits are higher with EAS-61 than with AS-1 because the hypotonic EAS leads to a 20% swelling of the RBCs with an equivalent increase in the cellular fractional volume.

Third study, AS-5, EAS-61 and EAS-64 units: Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CPD anticoagulant in the primary polyvinylchloride (PVC) bag of an AS-5 double-bag collection set (code 1BB*AGD456A, Terumo Medical Corp, Somerset, NJ). Packed cells were prepared by centrifugation at $5,000 \times g$ for 5 minutes at room temperature followed by the removal of sufficient plasma to achieve a hematocrit of 75%. Three units of identical ABO type were then pooled and realiquoted as described above, and either 100 mL of AS-5, 200mL of EAS-61, or 300 mL of EAS-64 was added.

All units were gently mixed by inversion 25 times, sampled aseptically for *in vitro* testing, and placed in refrigerated storage ($1-6^{\circ}\text{C}$) four hours or less after collection.

In vitro measurements

Samples from stored units were collected, after gentle mixing by inversion, by a sterile sampling procedure in a biological safety cabinet. A battery of *in vitro* tests was performed on all units at the beginning of storage and weekly thereafter.

For studies 1 and 3 performed at Walter Reed, the total hemoglobin (HGB) concentration, WBC counts, and RBC counts were measured with a clinical hematology analyzer (Hematology Cell Counter System Series 9000, Baker, Allentown, PA). Supernatant HGB was measured spectrophotometrically using the modified Drabkins assay.⁶ Percent hemolysis was determined by

the ratio of free to total hemoglobin. The results are expressed as percent hemolysis to compensate for the differences in hematocrit and Hgb values between samples. Centrifuged microhematocrits (Clay Adams, Becton-Dickinson, Rutherford, NJ) were performed to avoid the readjustment of cell volume and subsequent errors caused by the isotonic diluent used in the blood analyzer. Mean corpuscular volumes (MCV) were calculated from the spun microhematocrit and the RBC count. RBC morphology scores were determined according to the method of Usry, Moore, and Manalo.⁷

RBC ATP concentrations were measured in deproteinized supernatants. Whole blood or packed cell aliquots were mixed with cold 10% trichloroacetic acid to precipitate blood proteins, centrifuged at 2700 x g for 10 minutes, and the protein free supernatant frozen at -80°C until tested. ATP was assayed enzymatically using a commercially available test kit (Procedure 366-UV, Sigma Diagnostics, St. Louis, MO).

Supernatant electrolytes, pH, and blood gases were measured on a blood gas analyzer (Corning 855, Ithaca, NY). Thus, pH was measured at 37°C. Phosphate and glucose were measured on a programmable chemical analyzer (Cobas Fara, Roche Diagnostic Systems, Nutley, NJ)

For Study 2 performed at the Hoxworth Blood Center, a different clinical hematology analyzer was used (MaxM Coulter Counter, Coulter Electronics, Hialeah, FL). pH was measured with a benchtop pH meter (Orion 900A, Orion Research Inc., Boston, MA) at 22°C. Blood pH has a temperature coefficient of -0.015 pH units/°C, so pH measured at 22°C will be about 0.22 pH units higher than pH measured at 37°C. Supernatant potassium, glucose, and inorganic phosphorus testing was sent to an outside laboratory (Health Alliance Laboratories,

Cincinnati, OH). Inorganic phosphate was measured using a procedure by Boehringer Mannheim Corp., that involves the formation of ammonium phosphomolybdate (Hitachi 747-200). Supernatant hemoglobin was measured using 3,3',5,5'-tetramethylbenzidine (procedure No. 527, Sigma Diagnostics).

Statistical Analysis

Comparisons of means of measured values at given times within the individual cross-over trials were evaluated with the analysis of variance. Probabilities less likely than 0.05 were considered statistically significant.

Results:

Study 1. RBCs were stored in increasing volumes of AS-1. RBCs stored in the conventional 100 mL of AS-1 maintained RBC ATP concentrations close to their initial value of 5.2 $\mu\text{mol/g Hgb}$ for four weeks (Figure 1A). Thereafter, the RBC ATP concentration declined at -0.6 $\mu\text{mol/g Hgb/week}$. With increasing volumes of the additive solution, the initial RBC ATP concentration rose higher, to 5.5 and 5.8 $\mu\text{mol/g Hgb}$, but the subsequent decline began sooner, at 2½ and 1 weeks, respectively. However, the rate of decline remained the same at -0.6 $\mu\text{mol/g Hgb/week}$.

Supernatant pH was the same at each weekly measurement at all three suspension concentrations (Figure 1B). Inorganic phosphate, initially from the residual plasma and the CPD anticoagulant and later from the breakdown of ATP and 2,3-diphosphoglycerate, was diluted in the additional volumes of AS-1 (Figure 1C). The additional AS-1 volume decreased the RBC

hemolysis during storage by approximately 50% (Figure 1D).

Study 2. RBCs were stored in increasing volumes of EAS-61. RBC ATP concentrations were different after just one hour of storage, averaging $4.11 \pm .37$ $\mu\text{mol/g Hgb}$ with 100 mL of EAS-61, $4.54 \pm .34$ with 200, $4.96 \pm .15$ with 300, and $5.11 \pm .25$ $\mu\text{mol/g Hgb}$ with 400 mL (Figure 2A). Over the next two weeks these concentrations increased to $4.38 \pm .47$, $4.88 \pm .30$, $5.34 \pm .39$, and $5.56 \pm .39$ $\mu\text{mol/g Hgb}$, respectively. After that time, the RBC ATP concentration declined at a uniform rate of about -0.35 $\mu\text{mol/g Hgb/week}$ in all groups.

Supernatant pH was again the same at each weekly measurement (Figure 2B). Inorganic phosphate concentrations were all approximately 25 mg/dL (data not shown). The RBC morphology scores were all nearly 100% discocytes for all groups except for the 100 mL additive volume group (Figure 2C). The additive solution volumes of 200 mL or greater were also associated with a 50% reduction in RBC hemolysis (Figure 2D).

Study 3. RBCs were stored in 100 mL of AS-5, 200 mL of EAS-61, and 300 mL of EAS-64. RBC ATP concentrations were initially identical in all groups at about $4.8 \pm .3$ $\mu\text{mol/g Hgb}$. They remained stable in the AS-5 group for the next two weeks, but in the EASs they increased by about 10% over the first two weeks and remained elevated until 5 weeks (Figure 3A). In AS-5 the RBC ATP concentrations decreased after two weeks of storage at a steady rate of about -0.4 $\mu\text{mol/g Hgb/week}$. The same rate of decrease was observed with storage in the EASs, but the decrease started at 5 weeks of storage. Thus, the RBC ATP concentrations in the units stored in the experimental additive solutions were about 1 $\mu\text{mol/g Hgb}$ higher between five and 10 weeks of storage or remain above any given level for about 2½ to 3 weeks longer.

Supernatant pH decreased more rapidly in the first two weeks in the smaller volume of the

AS-5 system, and less rapidly in the later phases of storage (Figure 3B). Supernatant phosphate concentrations behaved very differently in the three storage solutions, rising continuously in AS-5, decreasing initially in EAS-61 and remaining relatively constant in EAS-64 (Figure 3C). Both of the EASs reduced RBC hemolysis by 75% (Figure 3D).

Discussion:

In 1986, Meryman, Hornblower and Syring showed that RBCs could be stored in experimental nutrient solutions for as long as 14 weeks and have *in vivo* 24 hour recoveries greater than 75%.⁵ Their storage solutions included conventional ingredients such as adenine, phosphate, citrate, and mannitol, but also unconventional ingredients such as ammonium and potassium salts. They also used unconventional formulations with low salt concentrations and neutral pH. The group was not able to discover which of the ingredients were responsible for the marked elevation of RBC ATP concentrations that seemed to be responsible for the prolonged viability.

Four laboratories have confirmed and attempted to extend the work of Meryman's group. In 1990, Greenwalt and his colleagues reported splitting units of RBCs and stored them, half in AS-1 and half in Meryman's solution 6.⁸ They were able to confirm the increased RBC ATP concentrations in the Meryman solution, but also showed that the low salt solution did not result in better membrane preservation. In 1992, this group reported that increasing amounts of disodium phosphate resulted in successive increases in RBC ATP concentrations.⁹ In 1993 they described acceptable *in vivo* RBC recoveries after 8 and 9 weeks of storage in 200 mL of an ammonium and phosphate containing additive solution, but stated that the ammonium would have

to be removed at the end of storage.¹⁰ In 1990, Mazor, Dvilansky, and Meyerstein showed that the ammonium and potassium in the Meryman solution 6 were not critical, as they could be replaced with sodium or rubidium with no difference in the initial rise in RBC ATP concentrations.¹¹ In 1994, they went on to show that the RBC ATP concentration was most directly affected by the pH and adenine and phosphate content.¹² In 1992, Kay and Beutler examined the mechanism by which Meryman's solution 6 increased stored RBC ATP concentrations.¹³ They concluded that the inhibition of phosphofructokinase by ATP was blocked by the ammonia in the solutions leading to higher than normal concentrations of ATP. Finally, Dumaswala and his colleagues compared SAG-M solutions supplemented with glutamine or glutamine and phosphate.¹⁴ The phosphate containing solution demonstrated increased RBC ATP concentrations.

The data from these studies did not directly address the effects of storage solution volume but suggested that it might be possible to make a directly infusable 200 mL 8- or 9-week RBC storage solution. We set out, therefore, to measure directly the effect of additive solution volume using two experimental and two conventional RBC storage solutions. In *Study 1*, increasing volumes of AS-1 resulted in lower RBC ATP concentrations but in less hemolysis in a dose-dependent manner. In *Study 2*, increasing volumes of EAS-61 resulted in increased RBC ATP concentrations and reduced hemolysis in a dose-dependent manner. In *Study 3*, AS-5, an AS-1-like SAG-M solution, and EAS-61 were directly compared along with a third 300 mL experimental additive solution, EAS-64. Storage in EASs again resulted in increased RBC ATP concentrations and reduced hemolysis during storage.

The observed effect of storage solution volume on RBC morphology and storage

hemolysis appears not to have been recognized previously. These findings are important, for if EAS-61 were used as an additive solution at the 100 mL volume, it would not meet the current U.S. Food And Drug Administration storage solution licensing requirements of less than 1% hemolysis during storage beyond 7 weeks.¹⁵ Nevertheless, the improvement in storage hemolysis observed with increasing volumes of EAS-61 solution appear to be greater than that observed with AS-1. This suggests that the reduced hemolysis is both an effect of the increased storage volume and the better maintenance of RBC energy metabolism. It is not obvious from the data why storage solution volume is important since the pH and concentration of inorganic phosphate, both known to be important for energy metabolism, were not correlated with hemolysis. Moreover, the cells, although mixed weekly for sampling, spend most of their time at high local concentrations in the bottom of the storage bag.

EAS-61 and EAS-64 appear to be a directly infusible RBC storage solutions. They are composed of saline, adenine, dextrose, mannitol, and disodium phosphate. These materials are already used in approved RBC storage solutions in the U.S. and/or Europe.

Conclusion

The data presented here suggest that EAS-61 and EAS-64 should be clinically tested to determine the adequacy of 24 hour *in vivo* RBC recovery after storage for 7, 8, 9 and 10 weeks. Longer lasting and better RBC storage will reduce RBC losses, improve the availability of blood in remote locations, and improve transfusion safety.

Acknowledgments:

The authors certify that they are employed, directly or indirectly, by the U.S. Army and the University of Cincinnati which have jointly filed for patent rights on the EAS-61 and EAS-64 experimental additive solutions. Two of the authors JRH and TJG are listed as inventors on the patent filing. This research was supported by the Combat Casualty Care Program of the U.S. Army Medical Research and Materiel Command

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Table 1 Composition of additive solutions used in this study (Concentrations in mMol).

	CPD	AS-1	AS5	EAS-61	EAS-64
Dextrose	142	111	45	110	50
Na ₃ Citrate	104				
Adenine		2	2.2	2	2
NaCl		154	150	26	75
Mannitol		41	45.5	55	20
Citric Acid	18				
Na ₂ PO ₄				12	9
pH	5.5	5.5	5.5	8.2	8.5

Figure Legends

Figure 1. The A) RBC ATP concentration, B) supernatant pH, C) supernatant inorganic phosphate concentration, and D) fractional hemolysis of pooled RBC units stored in the equivalent of 100 (-◆-), 200 (-■-), and 300 (-▲-) mL of AS-1 additive solution (n = 10 measurements at each point, data presented as mean ± SEM). The increasing volume of additive solution led to reduced RBC ATP concentrations in the later phases of storage despite equivalent pH and higher phosphate concentrations. The increased volume led to improved RBC integrity.

Figure 2. The A) RBC ATP concentration, B) supernatant pH, C) RBC morphology score, and D) fractional hemolysis of pooled RBC units stored in 100 (-◆-), 200 (-■-), 300 (-▲-), and 400 (-●-) mL of EAS6-1 additive solution (n = 6 measurements at each point, data presented as mean ± SEM). The increasing volume of additive solution led to increased RBC ATP concentrations in the later phases of storage despite equivalent pH. The increased volume led to improved RBC morphology scores and improved RBC integrity.

Figure 3. The A) RBC ATP concentration, B) supernatant pH, C) supernatant inorganic phosphate concentration, and D) fractional hemolysis of pooled RBC units stored in 100 mL of AS-5 (-◆-), 200 mL of EAS-61 (-■-), and 300 mL of ES-64 (-▲-) (n = 10 measurements at each point, data presented as mean ± SEM). The experimental additive solutions appear to maintain RBC ATP concentrations 2½ to 3 weeks longer and cause a marked reduction in RBC hemolysis during prolonged storage.

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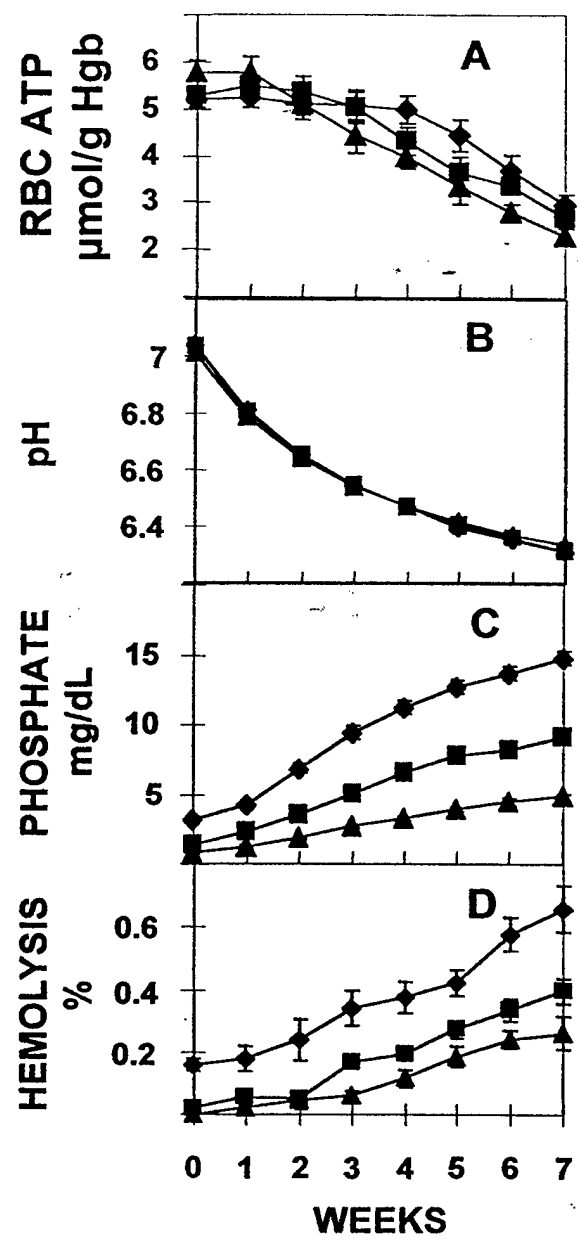
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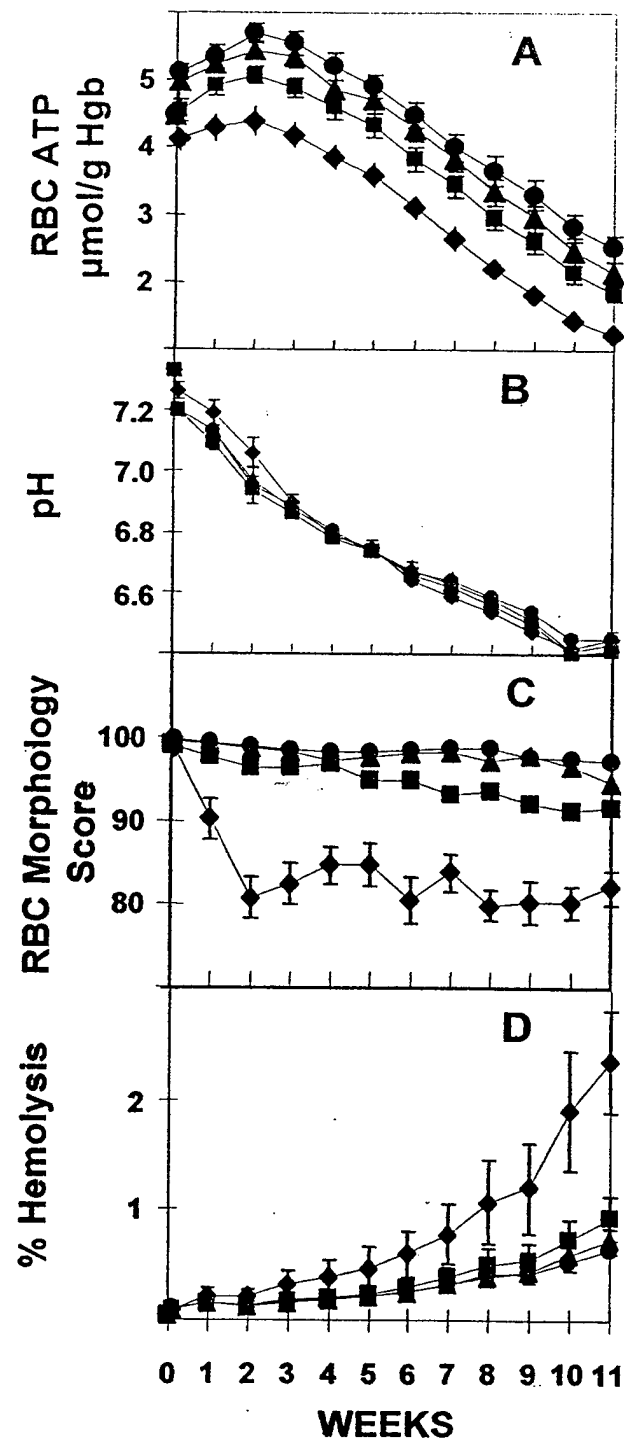
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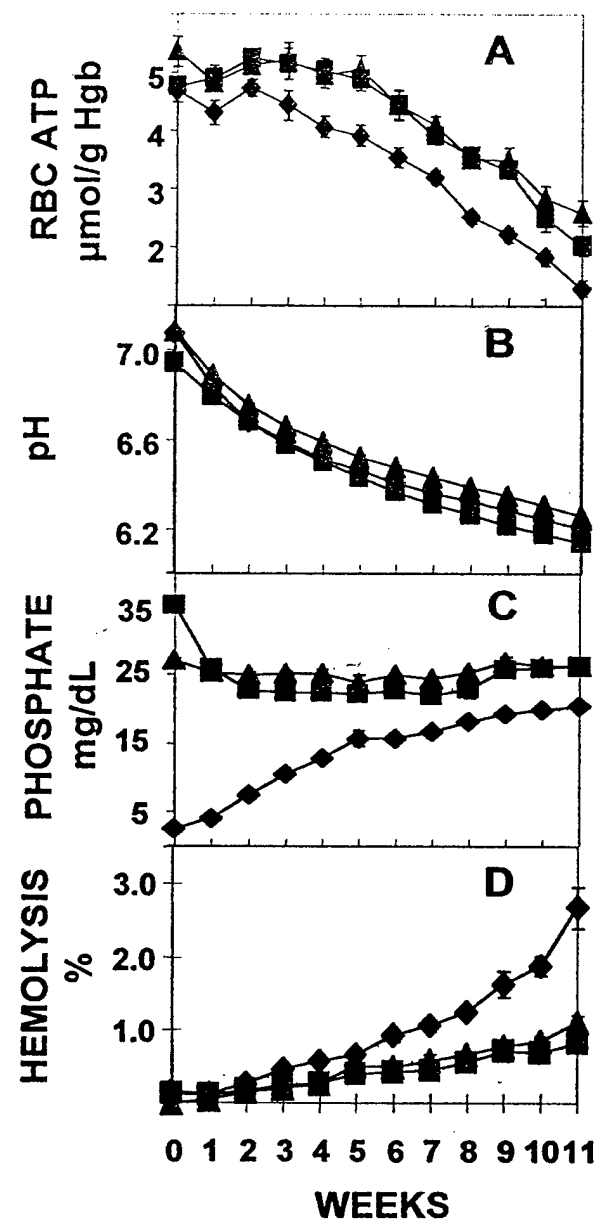
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Hess Fig 1







**A HYPOTONIC STORAGE SOLUTION DID NOT PROLONG THE VIABILITY OF
RED BLOOD CELLS**

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Running Head: RBCs stored in hypotonic solutions

Structured Abstract:

Background: Hypotonic storage solutions and leukofiltration are both reported to improve RBC viability. This study tested the ability of an investigational hypotonic storage solution, AS-24 (Medsep Corp), to extend the viability of liquid-stored RBC to 8 weeks.

Study design and Methods: In a pair of cross-over trials, 1) eleven RBC units, leukoreduced by filtration and stored in AS-24 for eight weeks were compared with units from the same donors stored for six weeks in AS-3, and 2) thirteen RBC units, leukoreduced by filtration and stored in AS-3 for eight weeks, were compared with units from the same donors stored for six weeks in AS-3. Viability was measured by the $^{51}\text{Cr}/^{99\text{m}}\text{Tc}$ double isotope method.

Results: RBC viability at 8 weeks averaged $64\pm3\%$ in the AS-24 units and $67\pm2\%$ in the AS-3 units, but was equal at $77\pm3\%$ and $77\pm2\%$ after 6 weeks storage in AS-3 in both trials.

Conclusions: Prestorage leukoreduction and storage in AS-24 did not extend RBC viability to 8 weeks. The improved viability previously demonstrated with storage of dilute suspensions of RBC in hypotonic solutions is probably caused by factors other than the hypotonicity.

Key Words: blood storage, RBC storage, humans, RBC viability, RBC survival, radioisotopes

Introduction:

Extending the shelf-life of liquid-stored red blood cells from 6 to 8 weeks has the potential to increase the availability of blood in remote areas and improve the utility of autologous blood storage for elective surgery.¹ Ideally, a RBC product with an extended shelf life should be physiologically compatible with transfusion in large volumes, retain conventional values of storage hematocrit and volume, and conform to standard manufacturing practices.

In 1986, Meryman, Hornblower, and Syring² suggested that increasing the membrane surface tension of stored RBCs would limit membrane loss by reducing the budding of microvesicles from surface spicules that develop on preserved cells. They reported that hypotonic solutions that induced RBC swelling *in vitro* stored RBC for more than 14 weeks with greater than 75% viability. Additional publications have supported this finding.³⁻⁹ However, these reports have two serious limitations. First, none of these studies examined the effect of hypotonic solutions alone in a system with a standard volume, a conventional hematocrit, or salts compatible with transfusion. Second, the majority of these studies relied on surrogates for viability as endpoints, such as RBC morphology and RBC ATP concentration.

This pair of studies was undertaken to determine the ability of a hypotonic storage solution, AS-24 (Medsep Corporation, Covina, CA), to extend the viability of RBC to 8 weeks. The AS-24 is a hypotonic solution of the type described by Meryman and his colleagues and was manufactured under a licence of their patent. The ability to extend viability was measured directly in a randomized cross-over study comparing the viability of RBCs stored in AS-24 for 8 weeks with the viability of RBCs from the same donors stored in an isotonic commercial additive solution, AS-3 (Nutricel®, Medsep Corporation, Covina, CA), for 6 weeks. In a parallel study

the viability of RBCs stored in AS-3 for 8 weeks was compared with their viability after storage in AS-3 for six weeks. In each cross-over study, the RBCs in the 8-week arm were leukoreduced by filtration in an attempt to further improve viability¹⁰, whereas the RBCs in the 6-week arm were not leukoreduced so as to provide a conventionally stored standard. The viability was measured 24 hours after reinfusion using the $^{51}\text{Cr}/^{99\text{m}}\text{Tc}$ double isotope technique.

Materials and Methods:

Volunteers

Thirty healthy volunteers meeting standard blood donor criteria¹¹ were enrolled in one or two studies after giving informed consent in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research and the U.S. Army's Human Subjects Research Review Board. There were thirty subjects in all with two subjects participating in both studies. Volunteers were tested to exclude sickle hemoglobin trait (Sicklescreen®, Pacific Hemostasis, Huntersville, NC) and abnormal osmotic fragility (Unopette™ Test 5830, Becton Dickinson, Rutherford, NJ).

Storage Solutions

The compositions of AS-3 and AS-24 are shown in Table 1. The significant differences in the solutions are the presence of mannitol and the absence of sodium chloride in the AS-24. Both solutions were formulated to have a pH of 5.8 and were used as 100 ml additive solutions for packed RBCs collected into CP2D.

Study Design

We conducted two parallel randomized cross-over studies. Each study allowed the direct comparison of the viability of 8-week stored RBCs compared to storage of cells from the same donor stored for 6 weeks in a standard, licensed additive solution. The results of the two studies were then compared. In the first cross-over study, RBC units were leukoreduced by filtration and stored in the investigational hypotonic medium AS-24 for eight weeks. They were compared with non-leukoreduced units from the same donors stored for six weeks in AS-3. In a second trial, RBC units were leukoreduced by filtration and stored in AS-3 for eight weeks. They were compared with second units stored for six weeks in AS-3. Electrolytes, pH, glucose, lactate, RBC morphology scores, and RBC ATP concentrations were measured at the beginning and end of storage. Viability was measured by the $^{51}\text{Cr}/^{99\text{m}}\text{Tc}$ double isotope method.¹² Post-reinfusion survival of the RBCs was measured as the RBC ^{51}Cr retention corrected for radioactive decay and 1% per day elution.

RBC Unit Preparation

Units of blood (450 ± 45 mL) were collected in CP2D anticoagulant and held for seven hours at room temperature.

AS-24 units (leukoreduced) held for eight weeks: Packed cells were prepared by centrifugation (5,000g, 5 min, room temperature (RT)) followed by the removal of plasma to achieve a target storage hematocrit of 65%. AS-24, 100 mL, was added followed by room temperature filtration to remove leukocytes (WBC) using an integral filter (Leukotrap® RC system, Medsep Corp., Covina, CA) and gravity flow.

AS-3 units (leukoreduced) held for 8 weeks: Whole blood was filtered at room temperature to remove WBC using an integral filter (Leukotrap®, Medsep) and gravity flow. Packed cells were prepared by centrifugation (5,000g, 5 min, RT) followed by removal of the plasma. Finally, 100 ml of AS-3 were added.

AS-3 units (non-leukoreduced) held for 6 weeks: Packed cells were prepared by centrifugation (5,000g, 5 min, RT) followed by removal of plasma to achieve a target storage hematocrit of 65%. Finally, 100 ml of AS-3 were added.

All units were gently mixed, sampled for *in vitro* testing, and placed in refrigerated storage (1-6°C) eight hours or less after collection.

In vitro measurements

Samples from stored units were collected into a small pouch attached to the residual donor needle tubing using a sterile connecting device (SCD 312, Terumo Medical Corporation, Elkton, MD). A battery of *in vitro* tests was performed on all units immediately before and after storage. Filtered units were also sampled prior to filtration.

Total hemoglobin (HGB) concentration, unfiltered WBC counts, and RBC counts were measured with a clinical hematology analyzer (Hematology Cell Counter System Series 9000, Baker, Allentown, PA). Supernatant HGB was measured spectrophotometrically (DU-62, Beckman, Fullerton, CA) using the modified Drabkins assay.¹³ Percent hemolysis was determined by the ratio of free to total hemoglobin. Centrifuged microhematocrits (Clay Adams, Becton-Dickinson, Rutherford, NJ) were performed to avoid the readjustment of cell volume and subsequent errors caused by the isotonic diluent used in the blood analyzer. Mean corpuscular

volumes (MCV) were calculated from the centrifuged microhematocrit and the RBC count. Post-filtration WBC counts were performed using a Neubauer hemocytometer and propidium iodide staining.¹⁴ The red cell morphology score was determined according to the method of Usry, Moore, and Manalo.¹⁵

RBC ATP concentration was measured in deproteinized supernatants. Whole blood or packed cell aliquots were mixed with cold, 12% perchloric acid to precipitate blood proteins, centrifuged at 2700 x g for 10 minutes, and the protein free supernatant was adjusted to pH 8-9 with solid KHCO_3 and frozen at -80°C until tested. ATP was assayed enzymatically using a commercially available test kit (Procedure 366-UV, Sigma Diagnostics, St. Louis, MO).

Check for Bacterial Contamination

Three or four days prior to the end of storage, sterilely collected aliquots from each unit were tested for bacterial contamination in broth and agar cultures using a commercial blood culture system (BBL Septi-Check™, Becton Dickinson Microbiology Systems, Cockeysville, MD). Absence of bacterial growth in the incubated cultures after 72 hours was required before the unit was prepared for reinfusion.

In Vivo Red Cell Recovery and Survival Measurement

After six or eight weeks of storage, *in vivo* RBC recovery was measured 24 hours after autologous reinfusion using a double radiolabel procedure. In brief, a sample of the stored blood was labeled with $20\mu\text{Ci } ^{51}\text{Cr}$. Concurrently, a fresh blood sample was collected from the volunteer and labeled with $25\mu\text{Ci } ^{99\text{m}}\text{Tc}$. Carefully measured aliquots of the radiolabeled red cells

were mixed and rapidly reinfused. Blood samples were collected at timed intervals during the 30 minutes immediately following the reinfusion and again at 24 hours, 7 days and 14 days post-reinfusion. Radioactivity of the samples was measured in a gamma counter (LKB CliniGamma Counter, Model 1272, Turku, Finland).

Gamma emissions from ^{99m}Tc radiolabeled cells were measured in the samples collected during the 30 minutes following reinfusion and used to determine an independent RBC volume. The activity from ^{51}Cr labeled cells was measured in all of the samples and used to calculate the fractional survival of the stored RBCs.

Statistical Analysis

Comparisons of means within the individual cross-over trials were evaluated with the paired *t* test. In comparisons between the cross-over trials, the unpaired *t* test was used. The correlations between morphology scores and viability and RBC ATP concentrations and viability were performed using the Pearson method. Probabilities less likely than 0.05 were considered statistically significant.

RESULTS:

Volunteers

Eleven volunteers completed the first study, and thirteen completed the second study. Two individuals participated in and completed both studies. The remaining eight volunteers withdrew for a variety of reasons, four related to the study. Two donors withdrew following unsuccessful phlebotomy, one after a tubing weld failure contaminated a unit, and one after

infiltration of a reinfusion site.

Prestorage Equivalence of Groups

Initial characteristics of the blood units are presented in Table 2. The units contained an average of 206 ± 12 mL of RBCs at collection. Leukoreduced units lost approximately 6% of their RBCs in the filter, and all units had 14 mL removed for prestorage testing. An additional 4mL was removed from the leukoreduced units to allow post-filtration leukocyte counting. Plasma was removed from the units to produce packed RBCs with a storage HCT of 65 %. The units began storage with RBC ATP concentrations averaging 5.1 ± 0.2 $\mu\text{mol/g}$ HGB and negligible hemolysis, equal in all groups.

Hypotonic Swelling

As indicated by a 14% increase in MCV from 95.7 ± 4.0 fL to the 109.5 ± 6.7 fL shown in Table 2, the AS-24 solution produced significant cell swelling. However AS-3, which is isotonic with an effective osmolality of 270 mOsm, also induced an increase in the MCV of the other units which averaged 8 fL. During the storage period, the AS-24 cells showed the greatest loss of volume, about 4%.

Leukofiltration

In the AS-24 units, the leukoreduction filter reduced the WBC from 6082/ μL to 0.63/ μL , a 4 log reduction to an average of 2.3×10^5 WBC per unit. The average time needed to filter the AS-24 units was 40 minutes because of the higher hematocrit of the RBC product and the size of

the swollen cells. The average filtration time for anticoagulated whole blood in the AS-3 units was 9 minutes, and the filter decreased WBC numbers below the detection limits of our assay (0.1 WBC/ μ L or 3.5×10^4 WBC per unit.). Despite the different preparative stages at which the AS-24 and AS-3 units were filtered, both groups lost only 6% of total red cells (23 gm of concentrated AS-24 product vs 35 gm of whole blood for the AS-3 units). Both groups achieved white cell reduction standards established in previous validation of the filters.

Post-storage Hemolysis, RBC ATP Concentrations, and RBC Morphology

Hemolysis averaged less than 1% under all conditions of storage in both studies (Table 3). Leukoreduced units in each study had significantly less hemolysis despite two weeks longer storage. RBC ATP concentrations were lower in the 8-week stored units in both studies than those measured with cells from the same donors stored for 6 weeks in AS-3. Despite the slight baseline differences in the two cross-over studies and two additional weeks storage for the 8-week stored units, there was no significant difference in the morphology scores of the four groups after the storage period.

Post-storage RBC viability

The 24 hour recovery fraction measures for the two studies are illustrated in Figure 1. The mean 24 hour survivals for the 6-week AS-3 "control" arm of each study were equivalent at 77% and greater than the FDA standard of 75%. The eight week arms of each trial had significantly lower survivals at $64 \pm 3\%$ for the AS-24 and $67 \pm 2\%$ for the AS-3 additive solutions. For all units, about 80% of the RBC surviving 24 hours persisted in the circulation for

fourteen days.

The relationship of RBC Morphology Score and RBC ATP Concentration to RBC viability.

An analysis of the relationship between the RBC morphology score and viability showed a significant correlation, $p = .001$. However, the correlation explained only 21% of the relationship (Figure 2). No morphology score defined a clear threshold for predicting adequate *in vivo* survival. RBC morphology scores of 60 or greater were counted for 9 of the 48 units; yet for 4 of these, the 24 hour survival values were below 75%. Likewise, of nineteen viability studies meeting or exceeding 75% recovery, morphology scores for 12 were below 60. The stored blood unit with the highest 24 hour recovery fraction, 95%, had a morphology score of only 55.

The correlation between RBC ATP concentration and RBC viability was stronger, accounting for 40% of the variability in the relationship (Figure 3). A $1 \mu\text{mol/g}$ HGB decrease in RBC ATP concentration at the end of storage is expected to reduce viability by $8.3 \pm 1.5 \%$ based on the observed regression coefficient. However, the positive predictive value of a RBC ATP concentration of $2 \mu\text{mol/g}$ HGB or greater was weak. Only 19 of the 41 units with RBC ATP concentrations meeting this criterion exhibited viability of 75% or greater, but all 7 units below that threshold demonstrated unacceptably low viability. Only 12 of 17 units with an RBC ATP concentration equal to or greater than $3 \mu\text{mol/g}$ HGB had a 24 hour survival of 75%.

Discussion:

In this investigation, we set out to test the prediction that a hypotonic additive solution would increase the viability of stored RBCs. Our paired cross-over study design produced

initially equivalent groups and the AS-24 storage solution did produce hypotonic swelling of the stored RBCs. Nevertheless, the viability was not usefully prolonged. Viability after 8 weeks storage in AS-24 appeared to be no better than with 8 weeks of storage in AS-3 solution when the RBCs were stored under otherwise equivalent conditions. Leukoreduction by filtration reduced RBC hemolysis by half during storage in the 8 week arms of both trials despite the 2 weeks longer storage. There appeared to be no additional benefit on reducing hemolysis conferred by storage in the hypotonic solution. Further, the RBC morphology scores of the leukoreduced units stored for 8 weeks were equal in the trials and equivalent to those of the non-leukoreduced units after six weeks of storage. In sum, the studies described here provide no evidence that the hypotonic additive solution improved viability, increased RBC morphology scores, or reduced hemolysis.

The 24-h recovery of the RBCs stored for 6 weeks in AS-3 are lower than in some previous reports,^{10,16} but they are not statistically different than those reported by Arduini and his colleagues.¹⁷ This lower-than-usual recovery appears to represent an unusually low subset within the range of viability measurements to be expected with a randomly chosen group of volunteers. The decrease in RBC ATP concentration with time between the end points of the 6- and 8-week arms was about 0.4 $\mu\text{mol/g}$ HGB/week and is typical of the decrease seen in other storage studies with repeated sampling of RBC units.¹⁸ The decrease in measured viability between 6 and 8 weeks of 10-13% is approximately what would be predicted from the decrease in RBC ATP concentration. The results suggest that the AS-24 solution is no better than AS-3 when used in the 100 ml additive solution format.

In their original description of hypotonic additive solutions, Meryman, Hornblower, and

Syring showed that RBCs, stored at a HCT of 35-40% with an initial pH of 7.1 in a hypotonic solution containing high concentrations of ammonium, potassium, phosphate, and membrane protectants such as mannitol and citrate, contained viable cells for as long as 19 weeks.² The paper was important because it showed that such extended storage was possible. The most successful of their solutions, solution "6," caused RBC ATP concentrations to rise to 160% of starting values at 4 weeks of storage. RBC ATP concentrations declined steadily thereafter at about 10% of the starting value each week, about 0.4 $\mu\text{mol/g}$ HGB/week.

Four groups have subsequently reexamined this concept. In 1990, Greenwalt and his colleagues confirmed the increased RBC ATP concentrations with storage in solution 6 in split-unit studies.^{5,19,20} Although they could also confirm better RBC morphology scores in the hypotonic media compared to storage in AS-1, they could not confirm RBC swelling by optical methods, and the loss of HGB in microvesicles was the same under both conditions of storage. Also in a 1990 report, Mazor, Dvilansky, and Meyerstein replaced the potassium and ammonium in Meryman's solution 6 with sodium and with rubidium, another monovalent cation, and found no difference in the initial rise in RBC ATP concentrations.⁶ In a 1994 report, the same group found that hypotonicity did not effect the RBC ATP concentration, but that initial pH, phosphate concentration, and adenine concentration were important.⁷ This group performed no studies of RBC viability. In 1992 Kay and Beutler also confirmed the ability of solution 6 to increase RBC ATP concentrations.⁸ They concluded that ammonium was important because it disinhibited phosphofructokinase. However, they could not demonstrate improved viability with storage of rabbit RBCs in solution 6. Finally, in 1996 Dumaswala and his colleagues reported a comparison of two hypotonic media, one with and one without phosphate.⁹ The less-hypotonic medium with

phosphate and a higher initial pH had better preservation of RBC ATP concentrations.

The demonstration of 14-week RBC storage by the Meryman group has always been clouded by the recognition that "from a clinical standpoint, solution number 6 would clearly be unacceptable for transfusion."² Attempts to understand and translate this work into clinically useful storage solutions have been limited by the failure to measure clinically relevant endpoints such as human RBC viability and storage hemolysis with reasonable statistical power. Future work in this field should attempt to maximize RBC ATP concentrations by the manipulation of pH, adenine, and phosphate content and minimize hemolysis by leukoreduction. Progress can be made with candidate additive solutions and storage systems incorporating these innovations.

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Table 1. Components of AS24 and AS-3 in millimoles and solution pH.

	AS-24	AS-3
Adenine	2	2
Dextrose	68.8	55.5
Mannitol	13.8	0
Na ₃ Citrate	17.7	20
NaH ₂ PO ₄	13	23
NaCl	0	70.1
H ₃ Citrate	3	2
pH	5.8	5.8

Table 2. Similarity of stored unit characteristics at the beginning of storage.

	First Study		Second Study	
	(N=11)		(N=13)	
	8 week	6 week	8 week	6 week
	Filtered	Unfiltered	Filtered	Unfiltered
	In AS-24	In AS-3	In AS-3	In AS-3
Hct	64.9±4.0	62.5±3.1	68.4±5.0	67.8±1.9
MCV	109.5±6.7*	101.2±4.6	103.2±7.0	101.3±3.2
ATP	5.1±0.3	5.1±0.2	5.0±0.5	5.2±0.7
Hemolysis	.026±.020	.016±.022	.014±.030	.012±.021
Morphology	93.4±3.1	93.5±3.6	89.2±4.0	88.7±3.5

Note: Data presented as Mean±SD. Only the MCV in the AS-24 units marked with the * was significantly greater than its paired control, the result of the hypotonic solution.

Table 3. Differences in stored unit characteristics at the end of storage

	First Study		Second Study	
	(N=11)		(N=13)	
	8 week	6 week	8 week	6 week
	Filtered	Unfiltered	Filtered	Unfiltered
	In AS-24	In AS-3	In AS-3	In AS-3
MCV	105.6±4.8*	100.1±5.3	100.9±4.2	100.3±3.7
ATP	2.0±.5*	3.1±.6	2.5±0.5*	3.2±0.6
Hemolysis	.31±.15*	.49±.31	.20±.07*	.51±.39
Morphology	53.7±4.5	55.8±5.3	52.6±4.6*	59.9±7.2

Note: Data presented as Mean ± SD. The values marked with * are significantly different from their paired controls at the $p < .05$ level. The lower hemolysis in the filtered units is probably clinically important as it occurred despite the units being stored for 2 weeks longer and having lower RBC ATP concentrations and recovery.

Figure Legends.

Figure 1. The distribution of RBC recovery measures in the two studies. The paired measures are connected and the mean values are presented as mean \pm SD.

Figure 2. The relationship of the RBC morphology index to *in vivo* RBC recovery fraction. Morphology is a poor surrogate for acceptable recovery.

Figure 3. The relationship of RBC ATP concentration to *in vivo* RBC recovery fraction. RBC ATP concentration is the most important *in vitro* correlate of RBC recovery, but both measures have large inherent inaccuracies of measurement which limits the value of individual measures of RBC ATP concentration.

